

The Next Generation of Monitoring Biologic Drug and Anti-Drug Antibody Levels

Tumor necrosis factor- α (TNF) is a proinflammatory cytokine implicated in the pathogenesis of chronic inflammatory disorders such as inflammatory bowel disease (IBD).^{1,2} Because of its central role in IBD pathogenesis, TNF is a logical target for therapeutic intervention, and the development of anti-TNF-directed therapies has been a major advance in the treatment of IBD. Monoclonal antibodies targeting TNF have proven highly effective in managing Crohn's disease (CD) and ulcerative colitis (UC). The anti-TNF agents—infliximab (IFX; Remicade[®], Janssen Biotech, Horsham, PA), a chimeric monoclonal antibody against TNF³⁻⁵; adalimumab (Humira[®], Abbott Laboratories, Abbott Park, IL), a fully human anti-TNF monoclonal antibody^{6,7}; and certolizumab pegol (Cimzia[®], UCB, Inc., Smyrna, GA), a humanized anti-TNF F(ab') monoclonal antibody fragment linked to polyethylene glycol^{8,9}—have demonstrated efficacy for induction and maintenance of remission in patients with moderate to severe CD or UC, or both.

Despite the substantial improvement in outcomes that anti-TNF agents have been able to provide for patients with IBD, response is not universal. More than one third of patients do not respond to induction therapy (primary nonresponse)^{6,8} and, even among initial responders, the response wanes over time in 20% to 60% of patients (secondary nonresponse).^{4,5,7,9,10} These therapeutic failures have not been addressed thoroughly and pose a challenge to clinicians. Hypotheses for treatment failure that are being actively investigated include the potential role of inadequate serum levels of the anti-TNF agent, consumption of the drug owing to high inflammatory disease burden, and development of immunogenicity (i.e., the formation of antibodies to the anti-TNF therapy) during the treatment course.^{11,12} Because of marked interindividual differences in drug pharmacokinetics, bioavailability, and immunogenicity, there is increasing recognition that optimizing treatment according to individual patient needs may provide a more rational therapeutic approach than universal use of a standard regimen derived from large clinical trials in nonuniform patient cohorts.¹¹ Emerging evidence indicates that higher serum drug levels are associated with longer durations of response and that development of antibodies-to-infliximab (ATI) is associated with shorter durations of response,¹³⁻¹⁵ suggesting that monitoring anti-TNF serum levels and anti-drug antibody status to inform treatment decisions may result in better outcomes for patients undergoing anti-TNF therapy. A recent review of clinical scenarios using anti-TNF serum levels and anti-drug antibody status to inform treatment decisions demonstrated the potential value of anti-TNF monitoring in clinical practice for optimizing individual treatment regimens.¹⁶ However, up to this point, the clinical utility of testing for these parameters has been limited by the sensitivity of currently available test methodologies, including the interference of serum anti-TNF drug levels with measurement of anti-drug antibodies.

This monograph focuses on IFX, the first anti-TNF agent available for the treatment of CD and UC, by presenting the following:

- A brief overview of the efficacy, safety, and tolerability of IFX in patients with IBD.
- Available evidence summarizing the interrelationship between serum IFX levels, ATI, and disease activity in patients who lose response during IFX therapy.
- A description of a newly available assay—PROMETHEUS[®] Anser[™] IFX (Prometheus Laboratories, San Diego, CA)—for the measurement of serum IFX and ATI concentration, including validation of the assay, its advantages over commonly used assays, and its clinical utility for improving the overall treatment of patients with IBD.

Infliximab

Infliximab is an intravenously administered chimeric immunoglobulin (Ig) G κ subclass monoclonal antibody that structurally consists of 75% human and 25% murine sequences.¹⁷ The variable murine region of infliximab was originally thought to confer immunogenicity (the potential for an antigen to induce an immune response after being recognized by a preexisting T-cell or B-cell receptor).¹⁸ However, all exogenous proteins are potentially immunogenic, and even fully human proteins can provoke an immune response. Many factors, in addition to degree of humanness, determine the immunogenicity of a biologic agent, including intrinsic patient characteristics, route of administration, and characteristics of the drug.¹⁸ IFX is approved for induction and maintenance of remission in patients with moderate to severe luminal or fistulizing CD refractory to conventional therapy, moderate to severe UC refractory to conventional therapy, and pediatric CD or UC refractory to conventional therapy.¹⁷ Other approved indications for IFX include rheumatoid arthritis, ankylosing spondylitis, psoriatic arthritis, and plaque psoriasis.¹⁷

Overview of Clinical Studies of IFX

A number of pivotal trials establishing the safety and efficacy of IFX in patients with IBD resulted in approval of this agent for treatment of patients with luminal or fistulizing CD, moderate to severe UC, and pediatric CD and UC. These studies are reviewed in brief, and the main results are presented here.

The safety and efficacy of single and multiple doses of IFX were assessed in two randomized, double-blind, placebo-controlled studies in patients with moderate to severe CD resistant to conventional therapy. Following single-dose administration, a clinical response was observed at 4 weeks in 81% of patients who received IFX 5 mg/kg, compared with 16% of placebo-treated patients ($P < .001$).¹⁷ The efficacy of repeated dosing for maintenance therapy

was subsequently demonstrated in the multidose A Crohn's Disease Clinical Trial Evaluating Infliximab in a new Long-Term Treatment Regimen I (ACCENT I) study in which 573 patients with CD received a single 5 mg/kg intravenous dose of IFX. Those who responded (335 [58%]) were randomly assigned at week 2 to one of the following regimens: placebo at weeks 2 and 6 and then every 8 weeks, IFX 5 mg/kg at the same time points, or IFX 5 mg/kg at weeks 2 and 6 followed by IFX 10 mg/kg every 8 weeks until week 46.^{4,17} In the week 2 responders, clinical remission was noted in 39% and 45% of patients who received IFX 5 mg/kg only or IFX 5 mg/kg followed by IFX 10 mg/kg, respectively, compared with 21% of patients who received placebo.⁴

The efficacy of IFX for treating fistulizing CD was demonstrated in two randomized, placebo-controlled trials in patients with fistulas of at least 3 months' duration.¹⁷ In the first trial, a fistula response after induction was observed in 68% and 56% of patients receiving IFX 5 mg/kg and IFX 10 mg/kg, respectively, compared with 26% of patients receiving placebo.^{17,19} In the second trial, ACCENT II,^{17,20} maintenance therapy with IFX was evaluated. In this trial, 306 patients with draining fistulas received IFX induction (5 mg/kg at weeks 0, 2, and 6). Of these, 282 were available for assessment of fistula response and 195 met fistula response criteria at weeks 10 and 14. Fistula responders were randomized to maintenance treatment with either placebo or IFX 5 mg/kg every 8 weeks through week 46. The time to loss of response among these patients (primary end point) was significantly longer in the IFX maintenance group (40 weeks) than in the placebo group (14 weeks; $P < .001$), and the fistula closure rate at week 54 was 36% with IFX maintenance compared with 19% with placebo ($P = .009$).²⁰

Two large placebo-controlled studies (Acute Ulcerative Colitis Treatment [ACT] 1 and ACT 2)^{5,17} evaluated the efficacy of IFX as induction and maintenance in patients with moderate to severe refractory UC. Patients were randomly assigned to receive placebo, IFX 5 mg/kg, or IFX 10 mg/kg at weeks 0, 2, 6, and every 8 weeks thereafter through week 22 (ACT 2) or week 46 (ACT 1). In ACT 1, a clinical response at week 54 was observed in 20%, 45%, and 44% of patients treated with placebo, IFX 5 mg/kg, or IFX 10 mg/kg, respectively; 17%, 35%, and 34% of patients were in clinical remission.^{5,17} Similarly, in ACT 2, a clinical response at week 30 was observed in 26%, 47%, and 60% of patients who received placebo, IFX 5 mg/kg, or IFX 10 mg/kg, respectively, with 11%, 26%, and 36% of patients achieving clinical remission.^{5,17}

In pediatric patients with CD, a randomized, open-label study showed that induction therapy with IFX 5 mg/kg at weeks 0, 2, and 6 resulted in a clinical response in 88% of patients and remission in 59% of patients at week 10.^{17,21} The response rate was similar to that reported in the adult CD trial. Subsequently, responders were

randomly assigned to a maintenance regimen of IFX 5 mg/kg administered either every 8 weeks or every 12 weeks. Treatment every 8 weeks was more effective than treatment every 12 weeks as measured by rate of sustained response (64% vs 33%) and clinical remission rate (56% vs 24%) at week 54.^{17,21}

Trials of IFX in adults with UC were used to support the efficacy and safety of IFX in pediatric patients with UC.^{17,22} Additional support for the use of IFX in pediatric patients with UC was provided by the results of an open-label study in which 44 of 60 (73.3%) pediatric patients with UC had a clinical response at week 8 after IFX induction; 24 of these patients were in clinical remission.^{17,22} At week 54, clinical remission was achieved in 8 of 21 (38.1%) patients and 4 of 22 (18.2%) patients who received maintenance treatment every 8 or 12 weeks, respectively.^{17,22}

In controlled clinical trials across all approved indications, the main adverse events reported more frequently with IFX than with placebo treatment were infections, infusion reactions, formation of autoantibodies, hypersensitivity, worsening of existing heart failure, malignancies (mainly lymphoma), and hepatotoxicity.¹⁷ Concern has been raised about serious infections resulting in death or hospitalization, including tuberculosis, bacterial sepsis, and invasive fungal and other opportunistic infections.¹⁷ Fatal cases of hepatosplenic T-cell lymphoma were reported post marketing in adolescent and young adult male patients with CD or UC who received IFX concurrently with immunosuppressants.¹⁷ The types and frequencies of adverse reactions associated with IFX treatment were similar across the various indications, with the exception of a higher incidence of abdominal pain reported in patients with CD.¹⁷ Similar to adverse reactions in adult CD, infections, infusion reactions, and hepatotoxicity were reported with IFX therapy in pediatric patients with CD, but the frequency of hematologic abnormalities (eg, anemia, neutropenia), flushing, viral and bacterial infections, fractures, and respiratory allergic reactions was higher in pediatric patients. In patients with UC, adverse events following IFX treatment were similar in both adult and pediatric populations.¹⁷

While IFX is efficacious and associated with improved outcomes in patients who were otherwise refractory to conventional agents, it is clear that not all patients with IBD respond to IFX and a proportion lose response over time. In trials reporting the use of IFX in patients with IBD, the rate of primary nonresponse was 12% to 44%^{3-5,19-21} and the rate of secondary nonresponse was 36% to 67%.^{5,20,21} Since its approval for use in patients with IBD, many subsequent investigations of IFX have been aimed at determining the optimal treatment regimen and dose schedule for IFX, along with optimal management of primary and secondary nonresponse. Available evidence on the impact of ATI formation, serum IFX levels, and concurrent immunosuppression on the efficacy and safety of IFX is reviewed here.

Table 1. Summary of Selected Studies of Antibodies-to-Infliximab, Infliximab Levels, and Clinical Outcomes. [Adapted with permission from Chaparro et al.²⁸]

Study	N	Indication	Regimen; Follow-up (median)	Patients With ATI	IFX Serum Levels	Impact of ATI on Safety	Impact of ATI on Efficacy	Impact of ATI on IFX Levels	Impact of IFX Levels on Efficacy
Crohn's Disease									
Baert et al ¹³	125	Refractory luminal or fistulizing CD	Episodic; 36 mo	61% overall (37% with ATI ≥ 8 $\mu\text{g/mL}$); 43% with IMM vs 75% without IMM	Higher levels in patients with IMM ($P < .01$)	ATI ≥ 8 $\mu\text{g/mL}$ predictive of infusion reaction (OR, 2.4); no direct comparison between ATI-positive vs ATI-negative patients	Shorter duration of response in patients with ATI; no direct comparison between ATI-positive vs ATI-negative patients; no significant influence of IMM	Lower levels in patients with ATI	Greater efficacy in patients with levels ≥ 12 $\mu\text{g/mL}$
Maser et al ¹⁴	105	Refractory inflammatory and/or perianal fistulizing CD	Episodic or scheduled; 23 mo	21% overall; 39% with episodic vs 16% with scheduled; IMM impacted ATI formation only with episodic therapy	IMM did not influence IFX levels	Higher infusion reaction rate in ATI-positive patients (50%) than ATI-negative patients (21%; $P < .05$); not affected by IMM	No association among ATI positivity and remission, CRP level, or endoscopic improvement	Not reported	Higher proportion of patients with detectable IFX levels achieved clinical remission (82% vs 6%; $P < .001$)
Hanauer et al ⁴	573	Moderate to severe CD	Scheduled (+ episodic retreatment); 54 wk	14% overall 28% placebo 9% IFX 5 mg/kg 6% IFX 10 mg/kg	Not reported	More infusion reactions in ATI-positive patients (38%) than in ATI-negative patients (24%)	No direct comparison between ATI-positive and ATI-negative patients	Lower IFX levels in ATI-positive patients	Not reported
Van Assche et al ²⁷	80	Moderate to severe CD and received episodic or scheduled IFX + azathioprine for 6 mo	Scheduled IFX, IMM continued or discontinued after 6 mo; 2 yr	Continued IMM: 5%; discontinued IMM: 12.5% ($P = .43$)	Not reported	Not reported	No association between ATI positivity and clinical response	Not reported	Lower IFX levels correlated with higher CRP levels and higher CDAI score
Afif et al ¹⁶	155	Patients with CD, UC, or indeterminate colitis who received IFX and were tested for IFX and ATI	93% scheduled; 50 wk	14% vs 29% with and without IMM; $P < .05$	Therapeutic levels in 48% vs 21% patients with and without IMM; $P < .001$	No direct comparison	No direct comparison; 17% of patients with loss of response vs 38% of patients with hypersensitive reactions were ATI positive	Not reported	No direct comparison; nontherapeutic levels found in 45% of patients with loss of response vs 20% of patients with acute reactions
Ulcerative Colitis									
Rutgeerts et al ⁵	364 (ACT 1) 364 (ACT 2)	Refractory moderate to severe UC despite concurrent corticosteroid and/or IMM treatment	Scheduled through wk 22 (ACT 2) or wk 46 (ACT 1); wk 30 (ACT 1), wk 54 (ACT 2)	6.1% (ACT 1) 6.4% (ACT 2)	Not reported	Higher infusion reaction rate in ATI-positive patients than in ATI-negative patients (ACT 1: 35.7% vs 9.8%; ACT 2: 50% vs 9.7%)	Similar rates of clinical response in ATI-positive and ATI-negative patients but higher rate in ATI-inconclusive patients	Not reported	Not reported
Seow et al ¹⁵	115	Moderate to severe UC	Scheduled; 13.9 mo	Overall: 41%; with IMM: 40%	39% with detectable levels	No difference in outcomes between ATI-positive and ATI-negative patients, but outcomes were superior in ATI-inconclusive patients	77% of patients with undetectable IFX levels had ATI	Detectable IFX levels were associated with increased clinical remission rate (69% vs 15%), increased endoscopic remission rate (27% vs 8%), and reduced colectomy rate (7% vs 55%)	

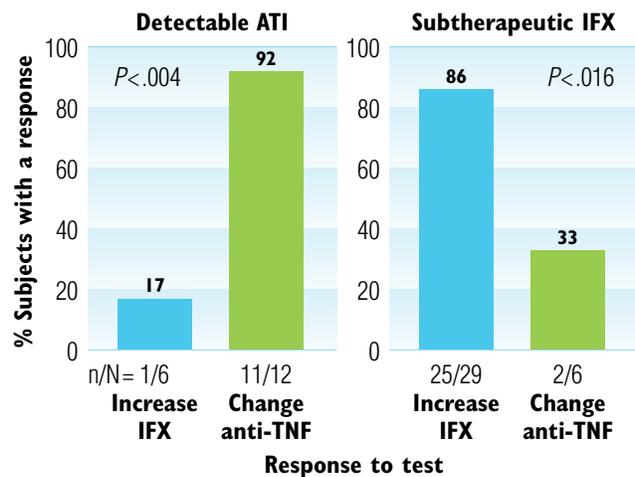
ACT, Active Ulcerative Colitis Trials; ATI, antibodies-to-infliximab; CD, Crohn's disease; CDAI, Crohn's Disease Activity Index; CRP, C-reactive protein; IFX, infliximab; IMM, immunomodulator therapy; OR, odds ratio; UC, ulcerative colitis.

Maser et al¹⁴ reported the findings from a prospective study in 105 patients with refractory inflammatory or fistulizing CD who were retreated either episodically at relapse (n=23) or on a scheduled regimen at 6- to 8-week intervals (n=82). Disease activity was measured with the Harvey-Bradshaw Index. After a median of 14 infusions, 21% of patients (22/105) had detectable ATI levels, 25% (26/105) were ATI negative, and 54% (57/105) had an inconclusive ATI status owing to the enzyme-linked immunosorbent assay (ELISA) assay drug and antibody interference limitation. The incidence of ATI formation in patients with CD was significantly lower with scheduled maintenance therapy than with episodic therapy (16% vs 39%; $P=.036$). On logistic regression analysis (ie, examination of gender, disease location, smoking status, indication for IFX, induction protocol, number of infusions, use of prednisone or immunomodulators, and maintenance regimen), the only variable that reduced the development of ATIs was a scheduled maintenance regimen (odds ratio [OR], 0.23; 95% CI, 0.08–0.73; $P=.012$). Infusion reactions occurred significantly more frequently in ATI-positive patients than in ATI-negative or ATI-inconclusive patients (50% vs 21%; OR, 3.6; $P=.018$). Serum concentrations of IFX were positively related to the interval of clinical remission (coefficient of determination [R^2]=0.61; $P<.001$) and change in endoscopic score ($R^2=0.46$; $P<.001$) and inversely related to serum CRP level ($R^2=0.26$; $P<.001$). In patients who continued with regular maintenance therapy, clinical remission was associated with detectable trough concentrations of IFX (82% vs 6% with undetectable trough levels; $P<.001$). Outcomes were not improved further with immunosuppressive therapy. A detectable trough concentration of serum IFX was the only significant predictor of improved outcomes.

In a recent report from the Mayo Clinic,¹⁶ the records of 155 patients who had ATI and IFX concentrations measured were reviewed to determine whether these data influenced patient management. One hundred twenty-seven patients (82%) received induction followed by scheduled dosing. The median time to initial testing after IFX initiation was 50 weeks, and each patient received a median of 8 infusions before testing. Initial complete and partial responses were achieved by 100 (65%) and 45 (29%) patients, respectively; 10 patients (6%) were nonresponders. ATIs were identified in 35 patients (23%), therapeutic IFX concentrations (defined as detectable IFX concentrations [>1.4 $\mu\text{g/mL}$] at trough or >12 $\mu\text{g/mL}$ 4 weeks after infusion) in 51 patients (33%), and subtherapeutic IFX concentrations (defined as undetectable at trough or <12 $\mu\text{g/mL}$ 4 weeks after infusion) in 69 patients (44%). Patients receiving concurrent immunosuppressive therapy (47%) were significantly more likely to have therapeutic levels of IFX than were those not receiving immunosuppressants (48% vs 21%; $P<.001$) and less likely to have ATI formation (14% vs 29%; $P<.032$). Of 177 tests assessed, the

results affected treatment decisions in 130 cases (73%). In ATI-positive patients, switching to another anti-TNF agent resulted in a partial or complete response in 11 of 12 patients (92%), whereas dose escalation was associated with a response in only 1 of 6 patients (17%; $P<.004$) (Figure 2). None of these patients developed therapeutic IFX concentrations with dose escalation. By contrast, among patients with subtherapeutic IFX levels, 86% (25/29) responded after dose escalation, whereas only 33% (2/6) responded after changing to another anti-TNF agent ($P<.016$). Among patients with clinical symptoms and therapeutic IFX concentrations, therapy was continued at the same dose in 76% of cases. This was the first study to show that measurement of ATI formation and IFX levels might be useful in making appropriate management decisions for patients with IBD.¹⁶

Figure 2. Management strategies and clinical outcomes in patients with detectable antibodies-to-infliximab (ATI) or subtherapeutic infliximab (IFX) concentrations.¹⁶



ATI, antibodies-to-infliximab; IFX, infliximab; TNF, tumor necrosis factor- α .

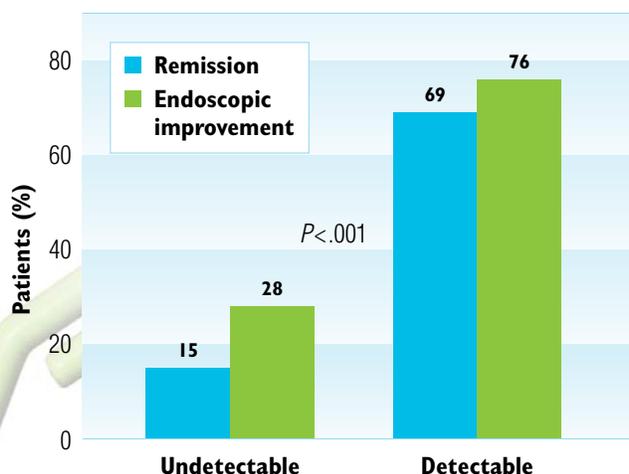
The development of immunogenicity to IFX in patients with UC was examined in two pivotal studies, ACT 1 and ACT 2.⁵ Both studies randomly assigned 364 patients with moderate to severe UC to receive infliximab 5 mg/kg, infliximab 10 mg/kg, or placebo at weeks 0, 2, and 6 and every 8 weeks thereafter through week 46 (ACT 1) or week 22 (ACT 2). Clinical response to IFX was defined as a decrease from baseline in the total Mayo score of ≥ 3 points and at least 30%, with an accompanying decrease in the subscore for rectal bleeding of at least 1 point or an absolute subscore for rectal bleeding of 0 or 1. Clinical remission was defined as a total Mayo score of ≤ 2 points, with no individual subscore exceeding 1 point.⁵

Baseline immunosuppressant use ranged from 44% to 55% in ACT 1 and from 42% to 44% in ACT 2.⁵ At week 54, among 229 patients in ACT 1, ATI status was positive, negative, or inconclusive in 14 (6%), 36 (16%), and 179 (78%), respectively; the corresponding

rates among 188 patients in ACT 2 were 12 (6%), 34 (18%), and 142 (76%), respectively. In ACT 1, 21% (3/14) of ATI-positive patients achieved a clinical response, compared with 8% (3/36) of ATI-negative patients and 58% (103/179) of ATI-inconclusive patients. In ACT 2, 58% (11/19) of ATI-positive patients showed a clinical response, compared with 57% (45/79) of ATI-negative patients and 77% (71/92) of patients with inconclusive ATI findings. In both studies, patients with ATIs were more likely to have an infusion reaction. At week 54 in ACT 1, infusion reactions developed in 36% (5/14) of ATI-positive patients, compared with 10% (21/215) of ATI-negative patients; in ACT 2 at week 30, infusion reactions developed in 50% (6/12) and 10% (17/176) of ATI-positive and ATI-negative patients, respectively.⁵

A study by Seow et al¹⁵ demonstrated an association between trough serum concentrations of IFX and clinical outcomes in patients with UC. In this cohort study, 115 patients with moderately severe to severe UC received a three-dose induction with IFX 5 mg/kg, and responders then received IFX infusions every 8 weeks. The median follow-up was 13.9 months. Of 108 patients, 66 had undetectable trough levels of IFX and, of these, 44 (41%) were ATI positive and 22 (20%) were ATI negative. The incidence of ATI formation was similar with or without the use of concurrent immunosuppression (40% vs 41%; $P=.88$). Patients with detectable serum IFX had a significantly higher rate of clinical remission (69% vs 15%; $P<.001$), endoscopic improvement (76% vs 28%; $P<.001$), and endoscopic remission (27% vs 8%; $P=.021$; **Figure 3**) and a lower rate of colectomy (7% vs 55%; $P<.001$). An undetectable trough IFX concentration was a significant predictor of the need for colectomy (OR, 9.3; 95% CI, 2.9–29.9; $P<.001$).¹⁵

Figure 3. Presence of detectable trough serum infliximab concentrations is associated with higher remission rate and endoscopic improvement. [Adapted with permission from Seow et al.¹⁵]



A post hoc analysis of data from the ACT 1 and ACT 2 trials also assessed the relationship between IFX levels and clinical outcomes.²⁸ At weeks 8, 30, and 54, median IFX concentrations were higher in patients achieving clinical response, remission, or mucosal healing than in those not achieving these outcomes. At these time points, rates of clinical remission increased with increasing quartiles of IFX concentrations. For example, at week 30, the median first, second, third, and fourth quartiles of IFX concentrations were <0.11 , ≥ 0.11 to <2.4 , ≥ 2.4 to <6.8 , and >6.8 $\mu\text{g/mL}$, respectively, with corresponding clinical remission rates of 15%, 26%, 60%, and 52% ($P<.0001$); similar trends were noted for clinical response and mucosal healing. As in the previously described study by Seow et al,¹⁵ higher IFX levels were associated with a greater likelihood of improved clinical outcomes.

The available evidence thus suggests that detectable IFX trough levels are associated with a longer duration of response and clinical remission. ATI formation and higher ATI levels are associated with loss of initial response and occurrence of infusion reactions, but this relationship is somewhat variable. The incidence of ATI formation reported in the literature varies, and ATI status, as measured with currently available assays, is inconclusive in a majority of patients,^{4,5,14} demonstrating a clear need for newer, more sensitive assays to measure both IFX levels and ATI in an effort to make them more clinically relevant for guiding treatment decisions.

Current Assays for Determining IFX Levels and Formation of ATI

Serum concentrations of IFX and ATIs are usually measured with ELISA. The most commonly used method to measure ATIs is a solid-phase (double-antigen or sandwich) ELISA in which ATIs in serum samples are captured on an IFX-coated solid phase through one F(ab') arm and are detected by binding of a biotinylated IFX conjugate through the other arm.¹³ However, there are limitations that may compromise the accuracy of this assay. Persistent IFX in the circulation can interfere with the detection of serum ATIs, leading to inconclusive results and potentially underestimating the presence of ATIs.^{27,28,30} This is especially problematic if sera are collected shortly after administration of IFX; most studies tried to minimize this problem by evaluating trough samples (measured just before the next IFX infusion). A potential confounder is the formation of immune complexes between IFX and ATIs, which limits detection of either IFX or ATIs and leads to false-negative results.^{31,32} Matrix effects may lead to epitope masking, which prevents detection of functionally monovalent immunoglobulins such as IgG4 and leads to false-negative results with bridging ELISA. A particular problem with solid-phase ELISAs is false-positive findings caused by nonspecific binding of other immunoglobulins, rheumatoid factors, or complement factors to the Fc segment of IFX.¹²

Radioimmunoassays (RIAs) have been developed in an attempt to overcome some of the drawbacks of solid-phase ELISAs.^{11,12,18,29,30} This assay avoids adherence of proteins to the solid surface and minimizes nonspecific binding of irrelevant IgG, is able to detect monovalent IgG4 as well as antibodies with weak affinities, and is less susceptible to artifacts from formation of neo-epitopes. However, RIA is technically more complex and requires disposal of radioactive waste.

To address the shortcomings and limitations of currently available assays, a new homogeneous mobility shift assay—Prometheus® Anser™ IFX Assay (Prometheus Laboratories, San Diego, CA)—has been developed to quantify both ATI and IFX levels simultaneously in

serum samples from patients with IBD. The development, analytical validation, and clinical use of the Prometheus Anser IFX Assay are described in the following sections.

Prometheus Anser IFX Assay

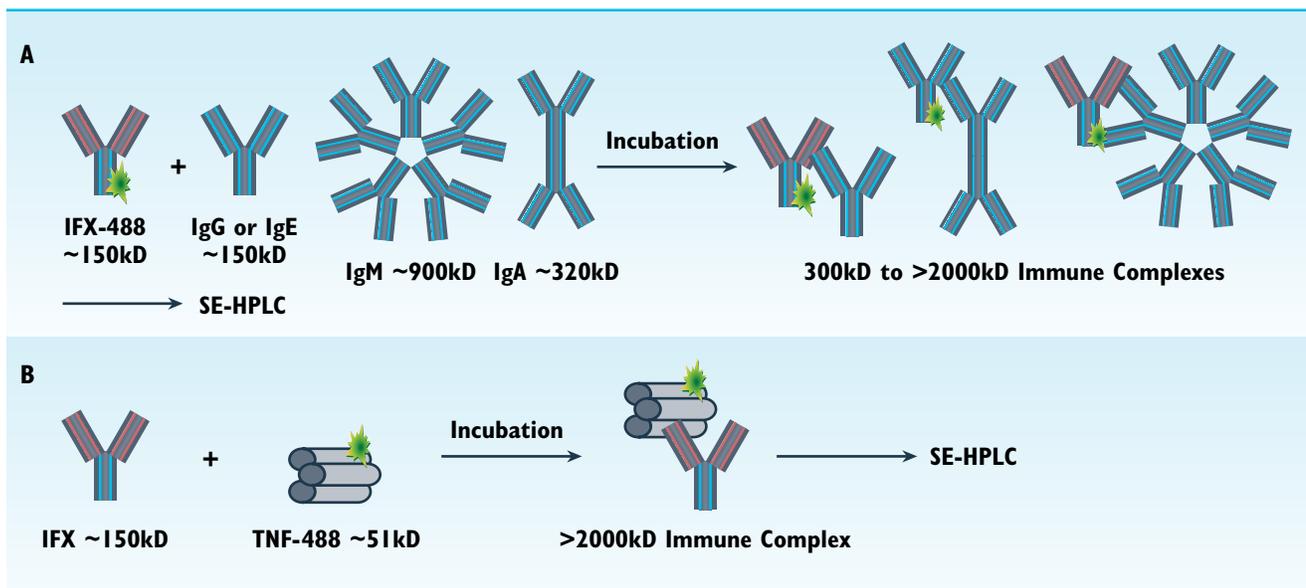
The Prometheus Anser IFX Assay is a new, proprietary, non-radiolabeled, fluid-phase mobility shift assay for the simultaneous detection of ATI and IFX. This assay offers several improvements over current ELISAs and RIAs; these are summarized in **Table 2**. The assay has a higher tolerance for IFX in the serum sample compared with current ELISAs and can measure both ATI and IFX in the same sample.

Table 2. Methods for Monitoring ATI Levels in Patient Serum Samples

	Bridging ELISA	Radio Immunoprecipitation Assay	Prometheus Anser IFX Assay
Nonspecific background interference	High	Low	Low
Sensitivity	Low	High	High
Possibility of false-positive or false-negative	High	Low	Low
IgG4 antibody detection	No	Yes	Yes
Ig isotope identification	No	No	Yes
Tolerance of drug in the sample	Poor	Good	Good
Use and disposal of radioactive material	No	Yes	No

ATI, antibodies-to-infliximab; ELISA, enzyme-linked immunosorbent assay; IFX, infliximab; Ig, immunoglobulin; IgG4, immunoglobulin G4.

Figure 4. Schematic illustration of the principles of the antibody (ATI) Prometheus Anser IFX Assay (A) and the infliximab (IFX) Prometheus Anser IFX Assay (B). [Reprinted with permission from Wang et al.³³]



IgA, IgE, IgG, and IgM, immunoglobulin A, E, G, and M; IFX, infliximab; SE-HPLC, size-exclusion high-performance liquid chromatography; TNF, tumor necrosis factor- α .

Principles

The principles of ATI and IFX level determination using the Prometheus® Anser™ IFX Assay are illustrated in Figure 4A and B. The ATI Prometheus Anser IFX Assay (Figure 4A) is based on the principle of a shift in the mobility of ATI bound to a fluorophore dye (Alexa Fluor 488)-labeled IFX (IFX-488) versus free IFX-488 because of the increased molecular weight of the complex.³³ The changes in the ratio of the internal control peak to the free IFX-488 peak are proportional to the amount of ATI. The amount of ATI in a sample is calculated from a standard curve generated from calibration standards using size-exclusion high-performance liquid chromatography (SE HPLC).³³

A similar principle applies to the IFX Prometheus Anser IFX Assay (Figure 4B). Incubation of fluorescently labeled TNF (TNF-488) with IFX leads to formation of higher-molecular-weight immune complexes (IFX–TNF-488). These are separated from free TNF-488 and quantified by SE-HPLC.³³

Analytical Validation of Prometheus Anser IFX Assay

Analytical validation of the Prometheus Anser IFX Assay showed that assay performance was robust for measurement of both ATI and IFX. To validate the standard curve, the performance characteristics of the ATI calibration standards within the concentration range of 0.006 to 0.720 µg/mL (0.033 to 4 U/mL) were monitored over 26 experiments by multiple analysts using different instruments over different days.³³ The quality of fit of the standard curve (residual sum of squares <0.001) was significantly better than the predetermined acceptability criterion of 0.01. The intra-assay and interassay precision rates (as indicated by the coefficient of variation [CV]) were <4% and <15%, respectively, which is considered to be highly robust. The lower and upper limits of quantitation (LLOQ and ULOQ, respectively) were the lowest and highest amounts of an analyte in a sample that could be quantitatively determined with suitable precision and accuracy. For the ATI Prometheus Anser IFX Assay, the LLOQ and ULOQ corresponded to effective serum concentrations of 0.56 and 27 µg/mL (3.1 and 150 U/mL), respectively.³³

The performance characteristics of the IFX Prometheus Anser IFX Assay standard curve in the concentration range of 0.03 to 3.75 µg/mL were similarly assessed in over 38 experiments by multiple analysts using different instruments on different days.³³ The intra-assay and interassay precision rates (CV) were <6% and <15%, respectively. The LLOQ and ULOQ corresponded to IFX serum concentrations of 0.98 and 34 µg/mL, respectively.³³ More recently, with optimization of the Prometheus Anser IFX Assay, the limit of quantitation of IFX has been lowered from 0.98 µg/mL to 50 ng/mL.³⁴ With the Prometheus Anser IFX Assay, IFX levels can be measured in the presence of ATI concentrations up to 18 µg/mL (100 U/mL), whereas with the ELISA, concentrations <1.8 µg/mL (10 U/mL) were disruptive.

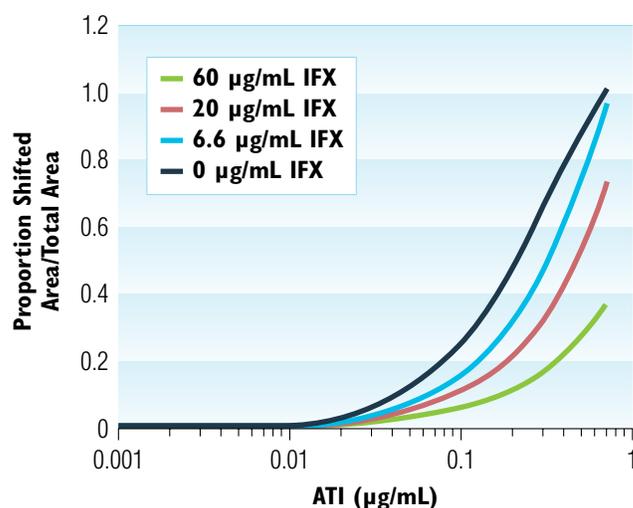
The cut point for the ATI Prometheus Anser IFX Assay, based on ATI levels in 100 serum samples collected from IFX-naïve healthy subjects, was 1.19 µg/mL (6.6 U/mL). The false-positive rate with this cut point was 3%.³³ Using the same samples, the cut point for the IFX Prometheus Anser IFX Assay was 0.98 µg/mL.³³

Potential Interference from Serum Components or IFX

Nonspecific binding of serum substances, which interferes with the accuracy of solid-phase ELISAs, is likely to be minimized in the fluid-phase Prometheus Anser IFX Assay. Potential interference in both ATI and IFX assays from common endogenous serum components was examined. There was no significant interference from physiologic levels of immunoglobulin, rheumatoid factor, hemolyzed serum, lipemic serum, azathioprine, and methotrexate in either assay, as assessed by the recovery of the ATI and IFX samples.³³

Because the presence of IFX in serum affects the detection of ATI in the current ELISA assay, it is important to evaluate the susceptibility of the new ATI Prometheus Anser IFX Assay to similar interference. The addition of increasing amounts of IFX (6.6, 20, and 60 µg/mL) to each of the 8 ATI calibration standards did not affect the standard curve.³³ As shown in Figure 5, the ATI Prometheus Anser IFX Assay detected ATI levels as low as 0.036 µg/mL in the presence of 60 µg/mL of IFX, which is much higher than the maximum therapeutic level attained after an IFX infusion.³³ Thus, the Prometheus Anser IFX Assay has a greatly improved tolerance for IFX and allows measurements of ATI concentrations in the presence of high IFX levels.

Figure 5. Antibody (ATI) Prometheus Anser IFX Assay drug tolerance. Prometheus Anser IFX Assay detects an ATI level as low as 0.036 µg/mL in the presence of an IFX level of 60 µg/mL. [Reprinted with permission from Wang et al.³³]



ATI, antibodies-to-infliximab; IFX, infliximab.

Clinical Validation of Prometheus Anser IFX Assay

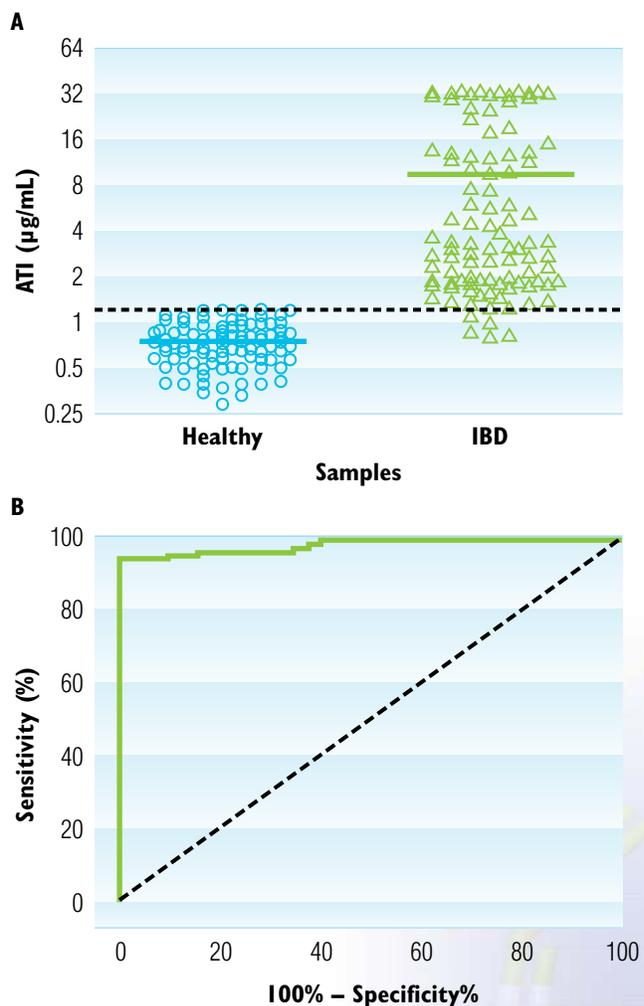
The bridging ELISA has been used over the past decade to measure ATIs in serum samples from IBD patients treated with IFX. To compare the performance of the ATI Prometheus Anser IFX Assay with the ELISA, 100 serum samples that previously tested positive with ELISA were reanalyzed with the ATI Prometheus Anser IFX Assay. The mean values of ATI in the patient serum samples were significantly higher than those in drug-naïve healthy controls (mean±SD=9.57±11.43 vs 0.73±0.29 µg/mL; $P<.0001$), as shown in **Figure 6A**.³³ Receiver operating characteristic (ROC) curve analysis of these samples showed that the area under the curve was 0.986 ± 0.007 (95% CI, 0.973–0.999; $P<.0001$), the sensitivity was 95% (95% CI, 88.7%–98.4%), with a specificity of 97%, and the OR was 47.5 when a 1.19-µg/mL cut point was used (**Figure 6B**).³³

The ATI Prometheus Anser IFX Assay also identified 5 false-positive patient samples from the ELISA; these samples tested negative with the Prometheus Anser IFX Assay most likely because of the higher rate of nonspecific binding in the ELISA method. By reducing nonspecific binding, the Prometheus Anser IFX Assay may provide greater specificity than the ELISA for detecting ATI.³³

The clinical utility of the Prometheus Anser IFX Assay has been examined in initial studies that have evaluated the relationship among serum IFX concentrations, ATIs, and disease outcomes. Feagan et al³⁵ analyzed 1,487 serum samples from 483 patients with CD who participated in 4 prospective randomized controlled trials or cohort studies of maintenance therapy with IFX. IFX and ATI were measured with the Prometheus Anser IFX Assay; CRP was used to assess disease activity. Paired samples obtained over sequential time points were evaluated for the relationship between presence of IFX and ATI in the first data point and CRP level in the subsequent measurement. ROC curve analysis showed that an IFX threshold concentration of 3 µg/mL best discriminated disease activity. Both ATI and IFX were related to the median CRP level (**Figure 7**). In a univariate analysis of the data, ATI-positive patients had significantly higher CRP levels than ATI-negative patients, even in the presence of high (≥ 3 µg/mL) IFX concentrations (11.57 vs 1.98, respectively; $P=.0002$). In ATI-negative patients, CRP was significantly lower in patients with IFX ≥ 3 µg/mL than with IFX < 3 µg/mL (1.98 vs 5.98 mg/L; $P<.0001$). In a multivariate regression analysis, positive ATI status was correlated significantly with CRP level, which was 59% higher in ATI-positive than in ATI-negative patients ($P=.0038$). For patients with IFX levels ≥ 3 µg/mL, CRP was 52% lower than in those with IFX levels < 3 µg/mL ($P=.0001$). These findings suggest that the benefits of IFX are diminished in the presence of ATI, even if drug concentrations are optimal.

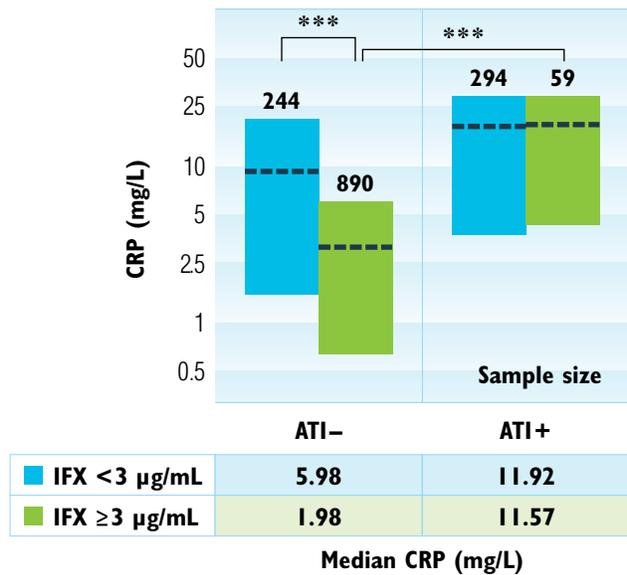
A study in pediatric patients with IBD showed that the Prometheus Anser IFX Assay could be used to measure IFX levels and ATI in this population as well.³⁶ Two hundred thirty serum samples from 71 children with IBD who received standard induction (weeks 0, 2, and 6) were tested. ATIs were detected in 47 of 230 samples ranging from 0.05 to >144 µg/mL (0.28 to >800 U/mL) from 21 children (30%). Eight of the 47 samples had measurable IFX (0.77 to 19.27 µg/mL), with a mean level of 12.36 µg/mL at infusion 3 and 6.53 µg/mL at infusion 4. Three children with Pediatric Crohn's Disease Activity Index score ≥ 25 at baseline had undetectable IFX and showed no clinical improvement at infusion 4.

Figure 6. (A) ATI concentrations in serum samples from patients with inflammatory bowel disease (IBD) and healthy controls, as determined by the Prometheus Anser IFX Assay. The horizontal dotted line represents the cut point and the horizontal solid line represents the mean. The y-axis scale is log₂. (B) Plot of the receiver operating characteristic curve using data from ATI Prometheus Anser IFX Assay analysis of serum samples from patients with IBD and healthy controls. [Reprinted with permission from Wang et al.³³]



ATI, antibodies-to-infliximab.

Figure 7. Disease activity in patients with Crohn's disease is linked to both ATI and IFX concentrations as measured with the Prometheus® Anser™ IFX Assay.³⁵



***P < .001.

ATI, antibodies-to-infliximab; CRP, C-reactive protein; IFX, infliximab.

Conclusions

IFX is an effective treatment for IBD, but the management of patients who demonstrate primary or secondary nonresponse is a therapeutic challenge. Several analyses have implicated low serum levels of IFX,¹³⁻¹⁵ which are variably associated with ATIs, in the loss of response to IFX. ATIs can cause adverse events such as infusion reactions, lead to ineffective therapeutic levels of IFX, and contribute to loss of response by increasing drug clearance or blocking the drug effect.^{5,13,14} Available evidence points to an interdependent relationship between ATI and IFX levels, suggesting that accurate measurement of both ATI and IFX during therapy should be an important consideration in the management of IFX treatment for IBD.^{16,23,24} However, methodologic problems associated with the measurement of ATIs and IFX levels when using the common ELISA has been one of the impediments to routine anti-TNF drug monitoring in clinical practice.^{12,27,28,30} The use of different assay techniques may have contributed to the inconsistent results noted among the studies.

The fluid-phase Prometheus Anser IFX Assay offers several advantages over the solid-phase bridge ELISA, the most commonly used assay for measuring ATIs and IFX levels in serum. The assay sensitivity is 18–45 times higher than the currently accepted sensitivity range achieved with the ELISA.³³ Importantly, the initial acid dissociation step of the Prometheus Anser IFX Assay increases tolerance for free IFX in the sample, allowing detection of low levels of ATI even in the presence of levels of IFX as high as 60 µg/mL.³³ By avoiding multiple washing steps, the Prometheus Anser IFX Assay can detect antibodies of low affinity, and lack of stereoscopic hindrance allows all immunoglobulin isotypes and subclasses of IgG to be detected. Nonspecific binding is limited because the antigen-antibody reaction occurs in the liquid phase, thereby reducing false-positive results.³³ Current research into the utility of the Prometheus Anser IFX Assay suggests that it is effective in measuring ATI in the presence of IFX in the serum of patients with IBD and, as a result, this assay may be more useful in informing treatment decisions than prior methodologies.

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Prometheus diagnostic services provide important information to aid in the monitoring and management of IBD. How this information is used to guide patient care is the responsibility of the physician.

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