ASSAYS FOR DETECTING AUTOANTIBODIES TO ANTI-TNFα DRUGS

~~CROSS-REFERENCE TO RELATED APPLICATIONS~~

~~[0001] This application claims priority to U.S. Provisional Application No. 61/444,097, filed February 17, 2011, U.S. Provisional Application No. 61/484,594, filed May 10, 2011, and U.S. Provisional Application No. 61/496,501, filed June 13, 2011, the disclosures of which are hereby incorporated by reference in their entirety for all purposes.~~

BACKGROUND OF THE INVENTION

[0002~~2~~1] Autoimmune disorders are a significant and widespread medical problem. For example, rheumatoid arthritis (RA) is an autoimmune disease affecting more than two million people in the United States. RA causes chronic inflammation of the joints and typically is a progressive illness that has the potential to cause joint destruction and functional disability. The cause of rheumatoid arthritis is unknown, although genetic predisposition, infectious agents and environmental factors have all been implicated in the etiology of the disease. In active RA, symptoms can include fatigue, lack of appetite, low grade fever, muscle and joint aches and stiffness. Also during disease flare ups, joints frequently become red, swollen, painful and tender, due to inflammation of the synovium. Furthermore, since RA is a systemic disease, inflammation can affect organs and areas of the body other than the joints, including glands of the eyes and mouth, the lung lining, the pericardium, and blood vessels.

Aarden et al describe the immunogenicity of anti-tumor necrosis factor antibodies-and provides improved methods of anti-antibody measurement (CURRENT OPINION IN IMMUNOLOGY, ELSEVIER, OXFORD, GB, vol. 20, no. 4, 1 August 2008, pages 431-435).

US 2009/035216 discloses a method for determining in vivo biopharmaceutical concentration and bioavailability.

Sickert et al. discuss the improvement of drug tolerance in immunogenicity testing by acid treatment on Biacore (JOURNAL OF IMMUNOLOGICAL METHODS, ELSEVIER SCIENCE PUBLISHERS B.V., AMSTERDAM, NL, vol. 334, no. 1-2, 20 May 2008, pages 29-36).

Lofgren et al. describes the detection of neutralizing anti-therapeutic protein antibodies in serum or plasma samples containing high levels of the therapeutic protein (JOURNAL OF

IMMUNOLOGICAL METHODS, ELSEVIER SCIENCE PUBLISHERS B.V.,AMSTERDAM, NL, vol. 308, no. 1-2, 20 January 2006, pages 101-108).

Bourdage et al. discloses an Affinity Capture Elution (ACE) assay for detection of anti-drug antibody to monoclonal antibody therapeutics in the presence of high levels of drug (JOURNAL OF IMMUNOLOGICAL METHODS, ELSEVIER SCIENCE PUBLISHERS B.V.,AMSTERDAM, NL, vol. 327, no. 1-2, 25 September 2007, pages 10-17).

Smith et al. describes the detection of antibodies against therapeutic proteins in the presence of residual therapeutic protein using a solid-phase extraction with acid dissociation (SPEAD) sample treatment prior to ELISA (REGULATORY TOXICOLOGY AND PHARMACOLOGY, ACADEMIC PRESS,NEW YORK, NY, US, vol. 49, no. 3, 13 November 2007, pages 230-237).

Patton et al. report an acid dissociation bridging ELISA for detection of antibodies directed against therapeutic proteins in the presence of antigen", JOURNAL OF IMMUNOLOGICAL METHODS, ELSEVIER SCIENCE PUBLISHERS B.V.,AMSTERDAM, NL, vol. 304, no. 1-2, 1 September 2005, pages 189-195).

Canan Abay et al. report the demonstration of specific antibodies against infliximab induced during treatment of a patient with ankylosing spondylitis (RHEUMATOLOGY INTERNATIONAL ; CLINICAL AND EXPERIMENTAL INVESTIGATIONS, SPRINGER, BERLIN, DE, vol. 26, no. 5, 1 March 2006, pages 473-480).

Bendtzen et al. describe the individualized monitoring of drug bioavailability and immunogenicity in rheumatoid arthritis patients treated with the tumor necrosis factor alpha inhibitor infliximab (ARTHRITIS & RHEUMATISM, JOHN WILEY & SONS, INC, US, vol. 54, no. 12, 1 December 2006, pages 3782-3789).

Rojas J.R. et al. report the formation, distribution, and elimination of infliximab and anti-infliximab immune complexes in cynomologus monkeys (JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS, AMERICAN SOCIETY FOR PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS, US, vol. 313, no. 2, 1 May 2005, pages 578-585).

[0001] Traditional treatments for the management of RA and other autoimmune disorders include fast acting “first line drugs” and slower acting “second line drugs.” The first line drugs reduce pain and inflammation. Example of such first line drugs include aspirin, naproxen, ibuprofen, etodolac and other non-steroidal anti-inflammatory drugs (NSAIDs), as

well as corticosteroids, given orally or injected directly into tissues and joints. The second line drugs promote disease remission and prevent progressive joint destruction and are also referred to as disease-modifying anti-rheumatic drugs or DMARDs. Examples of second line drugs include gold, hydrochloroquine, azulfidine and immunosuppressive agents, such as methotrexate, azathioprine, cyclophosphamide, chlorambucil and cyclosporine. Many of these drugs, however, can have detrimental side-effects. Thus, additional therapies for rheumatoid arthritis and other autoimmune disorders have been sought.

detect the presence of autoantibodies to anti-TNFα biologics in a patient sample to monitor TNFα inhibitor therapy and to guide treatment decisions. The present ~~invention~~ satisfies this need and provides related advantages as well.

BRIEF SUMMARY OF THE INVENTION

[0005] The present invention provides assays for detecting and measuring the presence or level of autoantibodies to anti-TNFα drug therapeutics in a sample. The present invention is useful for optimizing therapy and monitoring patients receiving anti-TNFα drug therapeutics to detect the presence or level of autoantibodies (*e.g.,* HACA and/or HAHA) against the drug. The present invention also provides methods for selecting therapy, optimizing therapy, and/or reducing toxicity in subjects receiving anti-TNFα drugs for the treatment of TNFα-mediated disease or disorders.

[0006] In one aspect, the present invention provides a method for detecting the presence or level of an autoantibody to an anti-TNFα drug in a sample without interference from the anti-TNFα drug in the sample, the method comprising:

(a) contacting the sample with an acid to dissociate preformed complexes of the autoantibody and the anti-TNFalpha drug, wherein the sample has or is suspected of having an autoantibody to the anti-TNFalpha drug;

(b) contacting the sample with a labeled anti-TNFalpha drug following dissociation of the preformed complexes, wherein optionally step (b) further comprises contacting a labeled internal control with the sample;

(c) neutralizing the acid in the sample to form labeled complexes of the labeled anti-TNFalpha drug and the autoantibody;

(d) subjecting the labeled complexes to size exclusion chromatography to separate the labeled complexes; and

(e) detecting the labeled complexes, thereby detecting the presence or level of the autoantibody without interference from the anti-TNFalpha drug in the sample.

The sample can be contacted with an acid at a concentration of from 0.1M to 5M.

The acid can be neutralized by adding one or more neutralizing agents to the sample.

The sample can be obtained from a subject receiving therapy with the anti-TNFalpha drug.

The complexes can be eluted first, followed by free labeled anti-TNFalpha drug.

The anti-TNFalpha drug can be labeled with a fluorophore or a fluorescent dye.

~~(a) contacting the sample with an acid to dissociate preformed complexes of the autoantibody and the anti-TNFα drug, wherein the sample has or is suspected of having an autoantibody to the anti-TNFα drug;~~

~~(b) contacting the sample with a labeled anti-TNFα drug following dissociation of the preformed complexes;~~

~~(c) neutralizing the acid in the sample to form labeled complexes (*i.e.*, immuno-complexes or conjugates) of the labeled anti-TNFα drug and the autoantibody (*i.e.*, wherein the labeled anti-TNFα drug and autoantibody are not covalently attached to each other);~~

~~(d) subjecting the labeled complexes to size exclusion chromatography to separate the labeled complexes (*e.g.*, from free labeled anti-TNFα drug); and~~

~~(e) detecting the labeled complexes, thereby detecting the presence or level of the autoantibody without interference from the anti-TNFα drug in the sample.~~

~~**[0009]** In some embodiments, the anti-TNFα drug is selected from the group consisting of REMICADE™ (infliximab), ENBREL™ (etanercept), HUMIRA™ (adalimumab), CIMZIA® (certolizumab pegol), SIMPONI® (golimumab; CNTO 148), and combinations thereof.~~

~~**[0010]** In other embodiments, the anti-TNFα drug autoantibody includes, but is not limited to, human anti-chimeric antibodies (HACA), human anti-humanized antibodies (HAHA), and human anti-mouse antibodies (HAMA), as well as combinations thereof.~~

[0011] In certain alternative embodiments, steps (a) and (b) are performed simultaneously, *e.g.*, the sample is contacted with an acid and a labeled anti-TNFα drug at the same time. In certain other alternative embodiments, step (b) is performed prior to step (a), *e.g.*, the sample is first contacted with a labeled anti-TNFα drug, and then contacted with an acid. In further embodiments, steps (b) and (c) are performed simultaneously, *e.g.*, the sample is contacted with a labeled anti-TNFα drug and neutralized (*e.g.*, by contacting the sample with one or more neutralizing agents) at the same time.

[0012] In particular embodiments, the sample is contacted with an amount of an acid that is sufficient to dissociate preformed complexes of the autoantibody and the anti-TNFα drug, such that the labeled anti-TNFα drug, the unlabeled anti-TNFα drug, and the autoantibody to the anti-TNFα drug can equilibrate and form complexes therebetween.

[0013] In another aspect, the present invention provides a method for optimizing therapy and/or reducing toxicity to an anti-TNFα drug in a subject receiving a course of therapy with the anti-TNFα drug, the method comprising:

(a) detecting the presence or level of an autoantibody to the anti-TNFα drug in a sample from the subject without interference from the anti-TNFα drug in the sample, the method comprising:

(i) contacting the sample with an acid to dissociate preformed complexes of the autoantibody and the anti-TNFα drug, wherein the sample has or is suspected of having an autoantibody to the anti-TNFα drug;

(ii) contacting the sample with a labeled anti-TNFα drug following dissociation of the preformed complexes;

(iii) neutralizing the acid in the sample to form labeled complexes (*i.e.*, immuno-complexes or conjugates) of the labeled anti-TNFα drug and the autoantibody (*i.e.*, wherein the labeled anti-TNFα drug and autoantibody are not covalently attached to each other);

(iv) subjecting the labeled complexes to size exclusion chromatography to separate the labeled complexes (*e.g.*, from free labeled anti-TNFα drug); and

(v) detecting the labeled complexes (*e.g.*, thereby detecting the presence or level of the autoantibody without interference from the anti-TNFα drug in the sample); and

~~(b) determining a subsequent dose of the course of therapy for the subject or whether a different course of therapy should be administered to the subject based upon the presence or level of the autoantibody,~~

~~thereby optimizing therapy and/or reducing toxicity to the anti-TNFα drug.~~

[0007] Methods for detecting anti-TNFα drugs and anti-drug antibodies are further described in PCT Publication No. WO 2011/056590~~, the disclosure of which is hereby incorporated by reference in its entirety for all purposes~~.

[0008] In other aspects, the present invention provides a method for selecting a course of therapy (*e.g.,* selecting an appropriate anti-TNFα drug) for the treatment of a TNFα-mediated disease or disorder in a subject, the method comprising:

(a) detecting the presence or level of an autoantibody to the anti-TNFalpha drug in a sample from the subject without interference from the anti-TNFalpha drug in the sample, the method comprising:

(i) contacting the sample with an acid to dissociate preformed complexes of the autoantibody and the anti-TNFalpha drug, wherein the sample has or is suspected of having an autoantibody to the anti-TNFalpha drug;

(ii) contacting the sample with a labeled anti-TNFalpha drug following dissociation of the preformed complexes;

(iii) neutralizing the acid in the sample to form labeled complexes of the labeled anti-TNFalpha drug and the autoantibody;

(iv) subjecting the labeled complexes to size exclusion chromatography to separate the labeled complexes; and

(v) detecting the labeled complexes to thereby detect the presence or level of the autoantibody without interference from the anti-TNFalpha drug in the sample; and

(b) determining a subsequent dose of the course of therapy for the subject or whether a different course of therapy should be administered to the subject based upon the presence or level of the autoantibody,

thereby optimizing therapy and/or reducing toxicity to the anti-TNFalpha drug.

The subsequent dose of the course of therapy can be increased, decreased, or maintained based upon the presence or level of the autoantibody.

The different course of therapy can comprise a different anti-TNFalpha drug, or wherein the different course of therapy comprises the current course of therapy along with an immunosuppressive agent, or wherein the different course of therapy comprises switching to a course of therapy that is not an anti-TNFalpha drug.

The anti-TNFalpha drug can be selected from the group consisting of infliximab, etanercept, adalimumab, certolizumab pegol, golimumab, CNTO 148, and combinations thereof.

The autoantibody to the anti- TNFalpha drug can be selected from the group consisting of a human anti-chimeric antibody (HACA), a human anti-humanized antibody (HAHA), a human anti-mouse antibody (HAMA), and combinations thereof.

The acid can comprise an organic acid, an inorganic acid, or mixtures thereof, wherein optionally the organic acid comprises citric acid.

~~(a) analyzing a sample obtained from the subject to determine the presence, level, or genotype of one or more markers in the sample;~~

~~(b) applying a statistical algorithm to the presence, level, or genotype of the one or more markers determined in step (a) to generate a disease activity/severity index; and~~

~~(c) selecting an appropriate course of therapy (*e.g.*, anti-TNFα therapy) for the subject based upon the disease activity/severity index.~~

~~**[0016]** In a related aspect, the present invention provides a method for optimizing therapy and/or reducing toxicity in a subject receiving a course of therapy for the treatment of a TNFα-mediated disease or disorder, the method comprising:~~

~~(a) analyzing a sample obtained from the subject to determine the presence, level, or genotype of one or more markers in the sample;~~

~~(b) applying a statistical algorithm to the presence, level, or genotype of the one or more markers determined in step (a) to generate a disease activity/severity index; and~~

~~(c) determining a subsequent dose of the course of therapy for the subject or whether a different course of therapy should be administered to the subject based upon the disease activity/severity index.~~

~~**[0017]** In particular embodiments, the methods of the present invention comprise detecting, measuring, or determining the presence, level (concentration (*e.g.*, total) and/or activation~~

~~(*e.g.*, phosphorylation)), or genotype of one or more specific markers in one or more of the following categories of biomarkers:~~

~~(1) Inflammatory markers~~

~~(2) Growth factors~~

~~(3) Serology (*e.g.*, immune markers)~~

(4) Cytokines and chemokines

(5) Markers of oxidative stress

(6) Cell surface receptors (*e.g.*, CD64, others)

(7) Signaling pathways

(8) Other markers (*e.g.*, genetic markers such as inflammatory pathway genes).

[0018] In further embodiments, the presence and/or level of one or both of the following markers can also be detected, measured, or determined in a patient sample (*e.g.*, a serum sample from a patient on anti-TNF drug therapy): (9) anti-TNF drug levels (*e.g.*, levels of free anti-TNFα therapeutic antibody); and/or (10) anti-drug antibody (ADA) levels (*e.g.*, levels of autoantibody to the anti-TNF drug).

[0019] In particular embodiments, a single statistical algorithm or a combination of two or more statistical algorithms can then be applied to the presence, concentration level, activation level, or genotype of the markers detected, measured, or determined in the sample to thereby generate the disease activity/severity index.

[0020] In certain instances, the sample is obtained by isolating PBMCs and/or PMN cells using any technique known in the art. In other embodiments, the sample is a tissue biopsy, *e.g.*, from a site of inflammation such as a portion of the gastrointestinal tract or synovial tissue.

[0021] Accordingly, in some aspects, the methods of the invention provide information useful for guiding treatment decisions for patients receiving or about to receive anti-TNFα drug therapy, *e.g.*, by selecting an appropriate anti-TNFα therapy for initial treatment, by determining when or how to adjust or modify (*e.g.*, increase or decrease) the subsequent dose of an anti-TNFα drug, by determining when or how to combine an anti-TNFα drug (*e.g.*, at an initial, increased, decreased, or same dose) with one or more immunosuppressive agents such as methotrexate (MTX) and/or azathioprine (AZA), and/or by determining when or how to change the current course of therapy (*e.g.*, switch to a different anti-TNFα drug or to a drug that targets a different mechanism such as an IL-6 receptor-inhibiting monoclonal antibody).

[0022] Other objects, features, and advantages of the present invention will be apparent to one of skill in the art from the following detailed description and figures.

BRIEF DESCRIPTION OF THE DRAWINGS

[0009] **Figure 1** shows an exemplary ~~embodiment of the~~ assays ~~of the present invention~~ wherein size exclusion HPLC is used to detect the binding between TNFα-$Alexa_{647}$ and HUMIRA™.

[0010] **Figure 2** shows dose response curves of HUMIRA™ binding to TNFα-$Alexa_{647}$.

[0011] **Figure 3** shows a current ELISA-based method for measuring HACA levels, known as the bridging assay.

[0012] **Figure 4** illustrates an exemplary outline of the autoantibody detection assays ~~of the present invention~~ for measuring the concentrations of HACA/HAHA generated against REMICADE™.

[0013] **Figure 5** shows a dose response analysis of anti-human IgG antibody binding to REMICADE™-$Alexa_{647}$.

[0014] **Figure 6** shows a second dose response analysis of anti-human IgG antibody binding to REMICADE™-$Alexa_{647}$.

[0015] **Figure 7** shows dose response curves of anti-human IgG antibody binding to REMICADE™-$Alexa_{647}$.

[0016] **Figure 8** shows REMICADE™-$Alexa_{647}$ immunocomplex formation in normal human serum and HACA positive serum.

[0017] **Figure 9** provides a summary of HACA measurements from 20 patient serum samples that were performed using the bridging assay or the mobility shift assay ~~of the present invention~~.

[0018] **Figure 10** provides a summary and comparison of current methods for measuring serum concentrations of HACA to the novel HACA assay ~~of the present invention~~.

[0019] **Figure 11** shows SE-HPLC profiles of fluorophore (Fl)-labeled IFX incubated with normal (NHS) or HACA-positive (HPS) serum. The addition of increasing amounts of HACA-positive serum to the incubation mixture dose-dependently shifted the IFX-Fl peak to the higher molecular mass eluting positions, C1 and C2.

[0020] **Figure 12** shows dose-response curves of the bound and free IFX-Fl generated with increasing dilutions of HACA-positive serum as determined by the mobility shift assay. (A) Increasing dilutions of HACA-positive serum were incubated with 37.5 ng of IFX-Fl. The

higher the dilution (less HACA) the more free IFX-Fl was found in the SE-HPLC analysis. (B) Increasing dilutions of HACA-positive serum were incubated with 37.5 ng of IFX-Fl. The higher the dilution (less HACA) the less HACA bound IFX-Fl was found in the SE-HPLC analysis.

[0021] **Figure 13** shows SE-HPLC profiles of TNFα-Fl incubated with normal (NHS) or IFX-spiked serum. The addition of increasing amounts of IFX-spiked serum to the incubation mixture dose-dependently shifted the fluorescent TNFα peak to the higher molecular mass eluting positions.

[0022] **Figure 14** shows dose-response curves of the bound and free TNFα generated with increasing dilutions of IFX-spiked serum as determined by the mobility shift assay. Increasing concentrations of IFX added to the incubation mixture decreases the percentage of free TNFα while increasing the percentage of bound TNFα.

[0023] **Figure 15** shows the measurement of relative HACA level and IFX concentration in IBD patients treated with IFX at different time points by the mobility shift assay.

[0024] **Figure 16** shows patient management- measurement of HACA level and IFX concentration in the sera of IBD patients treated with IFX at different time points.

[0025] **Figure 17** shows exemplary ~~embodiments of the~~ assays ~~of the present invention~~ to detect the presence of (A) non-neutralizing or (B) neutralizing autoantibodies such as HACA.

[0026] **Figure 18** shows ~~an alternative embodiment of the~~ assays ~~of the present invention~~ to detect the presence of neutralizing autoantibodies such as HACA.

[0027] **Figure 19** shows mobility shift profiles of Fl-labeled ADL incubated with normal human serum (NHS) in the presence of different amounts of anti-human IgG. The addition of increasing amounts of anti-human IgG to the incubation mixture dose-dependently shifted the free Fl-ADL peak (FA) to the higher molecular mass eluting positions, C1 and C2, while the internal control (IC) did not change.

[0028] **Figure 20** shows a dose-response curve of anti-human IgG on the shift of free Fl-ADL. Increasing amounts of anti-human IgG were incubated with 37.5 ng of Fl-ADL and internal control. The more the antibody was added to the reaction mixture the lower the ratio of free Fl-ADL to internal control.

[0029] **Figure 21** shows mobility shift profiles of Fl-labeled TNF-α incubated with normal human serum (NHS) in the presence of different amounts of ADL. Ex = 494 nm; Em = 519

[0043] **Figure 35** shows the analysis of samples containing TNF-Alexa488 and Remicade by mobility shift assay using a fluorescence detector with gain settings at different values.

[0044] **Figure 36** shows isoabsorbance plots taken for normal human serum (top panel) and TNF-Alexa488 (bottom panel) in HPLC mobile phase (1X PBS, 0.1% BSA in water). Excitation wavelengths are plotted on the Y-axis and emission wavelengths are plotted on the X-axis.

[0045] **Figure 37** shows the HPLC analysis of normal human serum (left) and 25ng TNF-Alexa488 (right) detected with indicated settings. The background level of fluorescence from normal human serum is greatly decreased.

[0046] **Figure 38** shows the standard curve generated by HPLC analysis of samples containing a fixed amount of TNF-Alexa488 and titrated with various amounts of Remicade.

[0047] **Figure 39** shows a comparison of Infliximab determination in clinical samples by mobility shift assay and ELISA. Dark grey points are for HACA-positive samples and light grey points are for HACA-negative samples. Dashed lines represent lower limits of quantitations for the respective methods.

[0048] **Figure 40** shows a comparison of HACA determination in clinical samples by mobility shift assay and ELISA.

[0049] **Figure 41** shows the cumulative counts of HACA-positive clinical samples as determined by mobility shift assay and ELISA.

DETAILED DESCRIPTION ~~OF THE INVENTION~~

I. Introduction

[0050] The present invention is based in part on the discovery that a homogeneous mobility shift assay using size exclusion chromatography and acid dissociation to enable equilibration of immune complexes is particularly advantageous for measuring the presence or level of autoantibodies (*e.g.,* HACA, HAHA, *etc.*) that are generated against anti-TNFα drugs. Such autoantibodies are also known as anti-drug antibodies or ADA. As a result, the presence or level of autoantibodies to an anti-TNFα drug administered to a subject in need thereof can be measured without substantial interference from the administered anti-TNFα drug that is also present in the subject's sample. In particular, a subject's sample can be incubated with an amount of acid that is sufficient to provide for the measurement of the presence or level of autoantibodies in the presence of the anti-TNFα drug without substantial interference from high anti-TNFα drug levels.

[0051] High anti-TNFα drug levels in a sample (*e.g.,* high infliximab levels) interferes with the measumrent of anti-drug antibody levels (*e.g.,* HACA levles). Under certain high drug conditions, the anti-drug antibody present in a sample is complexed with the unlabeled drug also present in the sample. When a labeled drug, *e.g.* labeled-infliximab, is contacted with the sample, the anti-drug antibody present in the sample is kinetically trapped from forming a complex with the labeled drug. In this way, the preformed complexes of anti-drug antibody and the unlabeled drug interfere with the measurement of anti-drug antibody, which depends on the formation of a complex between the anti-drug antibody present and the labeled drug. The acid dissociation step described herein allows for the anti-drug antibody present in the sample to dissociate from the unlabeled drug and reform complexes with both the labeled and unlabeled drug. By dissociating the anti-drug antibody from the unlabeled drug, the anti-drug antibody present in a sample can equilibrate between the labeled drug and the unlabeled drug.

[0052] As shown in Figure 27, high levels of anti-TNFα drug (*e.g.,* infliximab) interfere with the detection of anti-drug antibodies (*e.g.,* antibodies to infliximab or ATI) when the mobility shift assay is performed without an acid dissociation step. However, Figure 28 shows that acid dissociation followed by homogeneous solution phase binding kinetics to allow the equilibration and reformation of immune complexes significantly increased the anti-TNFα drug tolerance such that anti-drug antibodies can be measured in the presence of high levels of anti-TNFα drug (*e.g.,* up to or at least about 60 µg/mL). As such, the assays ~~of the present invention~~ are particularly advantageous over methods currently available because they enable the detection and measurement of anti-drug antibodies at any time during therapy with an anti-TNFα drug (*e.g.,* irrespective of low, medium, or high levels of anti-TNFα drug in a sample such as a blood sample), thereby overcoming a major limitation of methods in the art which require sample collection at trough concentrations of the drug.

[0053] In certain aspects, the present method ~~invention~~ is advantageous because it addresses and overcomes current limitations associated with the administration of anti-TNFα drugs such as infliximab, in part, by providing information useful for guiding treatment decisions for those patients receiving or about to receive anti-TNFα drug therapy. In particular, the methods ~~of the present invention~~ find utility for selecting an appropriate anti-TNFα therapy for initial treatment, for determining when or how to adjust or modify (*e.g.,* increase or decrease) the subsequent dose of an anti-TNFα drug to optimize therapeutic efficacy and/or to reduce toxicity, for determining when or how to combine an anti-TNFα drug (*e.g.,* at an initial, increased, decreased, or same dose) with one or more

immunosuppressive agents such as methotrexate (MTX) or azathioprine (AZA), and/or for determining when or how to change

the current course of therapy (*e.g.,* switch to a different anti-TNFα drug or to a drug that targets a different mechanism).

[0054] Accordingly, the present method ~~invention~~ is particularly useful in the following methods of improving patient management by guiding treatment decisions:

1. Crohn's disease prognostics: Treat patients most likely to benefit from therapy
2. Anti-therapeutic antibody monitoring (ATM) + Biomarker-based disease activity index
3. ATM sub-stratification
4. ATM with pharmacokinetic modeling
5. Monitor response and predict risk of relapse:
    a. Avoid chronic maintenance therapy in patients with low risk of recurrence
    b. Markers of mucosal healing
    c. Therapy selection: Whether to combine or not to combine anti-TNF drug therapy with an immunosuppressive agent such as MTX or AZA
6. Patient selection for biologics.

II. Definitions

[0055] As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

[0056] The terms "anti-TNFα drug" or "TNFα inhibitor" as used herein is intended to encompass agents including proteins, antibodies, antibody fragments, fusion proteins (*e.g.*, Ig fusion proteins or Fc fusion proteins), multivalent binding proteins (*e.g.,* DVD Ig), small molecule TNFα antagonists and similar naturally- or nonnaturally-occurring molecules, and/or recombinant and/or engineered forms thereof, that, directly or indirectly, inhibit TNFα activity, such as by inhibiting interaction of TNFα with a cell surface receptor for TNFα, inhibiting TNFα protein production, inhibiting TNFα gene expression, inhibiting TNFα secretion from cells, inhibiting TNFα receptor signaling or any other means resulting in decreased TNFα activity in a subject. The term "anti-TNFα drug" or "TNFα inhibitor" preferably includes agents which interfere with TNFα activity. Examples of anti-TNFα drugs include, without limitation, infliximab (REMICADE™, Johnson and Johnson), human anti-TNF monoclonal antibody adalimumab (D2E7/HUMIRA™, Abbott Laboratories), etanercept (ENBREL™, Amgen), certolizumab pegol (CIMZIA®, UCB, Inc.), golimumab (SIMPONI®; CNTO 148), CDP 571 (Celltech), CDP 870 (Celltech), as well as other compounds which inhibit TNFα activity, such that when administered to a subject suffering from or at risk of

suffering from a disorder in which TNFα activity is detrimental (*e.g.,* RA), the disorder is treated.

[0057] The term "TNFα" is intended to include a human cytokine that exists as a 17 kDa secreted form and a 26 kDa membrane associated form, the biologically active form of which is composed of a trimer of noncovalently bound 17 kDa molecules. The structure of TNFα is described further in, for example, Jones *et al*., *Nature*, 338:225-228 (1989). The term TNFα is intended to include human TNFα, a recombinant human TNFα (rhTNF-α), or TNFα that is at least about 80% identity to the human TNFα protein. Human TNFα consists of a 35 amino acid (aa) cytoplasmic domain, a 21 aa transmembrane segment, and a 177 aa extracellular domain (ECD) (Pennica, D. *et al.* (1984) *Nature* 312:724). Within the ECD, human TNFα shares 97% aa sequence identity with rhesus TNFα, and 71% to 92% aa sequence identity with bovine, canine, cotton rat, equine, feline, mouse, porcine, and rat TNFα. TNFα can be prepared by standard recombinant expression methods or purchased commercially (R & D Systems, Catalog No. 210-TA, Minneapolis, Minn.).

[0058] ~~In certain embodiments,~~ "TNFα" ~~is~~ can be an "antigen," which includes a molecule or a portion of the molecule capable of being bound by an anti-TNF-α drug. TNFα can have one or more than one epitope. In certain instances, TNFα will react, in a highly selective manner, with an anti-TNFα antibody. Preferred antigens that bind antibodies, fragments, and regions of anti-TNFα antibodies include at least 5 amino acids of human TNFα. In certain instances, TNFα is a sufficient length having an epitope of TNFα that is capable of binding anti-TNFα antibodies, fragments, and regions thereof.

[0059] The term "predicting responsiveness to an anti-TNFα drug" is intended to refer to an ability to assess the likelihood that treatment of a subject with an anti-TNFα drug will or will not be effective in (*e.g.*, provide a measurable benefit to) the subject. In particular, such an ability to assess the likelihood that treatment will or will not be effective typically is exercised after treatment has begun, and an indicator of effectiveness (*e.g.,* an indicator of measurable benefit) has been observed in the subject. Particularly preferred anti-TNFα drugs are biologic agents that have been approved by the FDA for use in humans in the treatment of TNFα-mediated diseases or disorders and include those anti-TNFα drugs described herein.

[0060] The term "size exclusion chromatography" or "SEC" includes a chromatographic method in which molecules in solution are separated based on their size and/or hydrodynamic volume. It is applied to large molecules or macromolecular complexes such as proteins and

[0066] The term "co-administer" includes to administer more than one active agent, such that the duration of physiological effect of one active agent overlaps with the physiological effect of a second active agent.

[0067] The term "subject," "patient," or "individual" typically refers to humans, but also to other animals including, *e.g.*, other primates, rodents, canines, felines, equines, ovines, porcines, and the like.

[0068] The term "course of therapy" includes any therapeutic approach taken to relieve or prevent one or more symptoms associated with a TNFα-mediated disease or disorder. The term encompasses administering any compound, drug, procedure, and/or regimen useful for improving the health of an individual with a TNFα-mediated disease or disorder and includes any of the therapeutic agents described herein. One skilled in the art will appreciate that either the course of therapy or the dose of the current course of therapy can be changed (*e.g.,* increased or decreased) based upon the presence or concentration level of TNFα, anti-TNFα drug, and/or anti-drug antibody using the methods ~~of the present invention~~.

[0069] The term "immunosuppressive drug" or "immunosuppressive agent" includes any substance capable of producing an immunosuppressive effect, *e.g.,* the prevention or diminution of the immune response, as by irradiation or by administration of drugs such as anti-metabolites, anti-lymphocyte sera, antibodies, *etc*. Examples of immunosuppressive drugs include, without limitation, thiopurine drugs such as azathioprine (AZA) and metabolites thereof; anti-metabolites such as methotrexate (MTX); sirolimus (rapamycin); temsirolimus; everolimus; tacrolimus (FK-506); FK-778; anti-lymphocyte globulin antibodies, anti-thymocyte globulin antibodies, anti-CD3 antibodies, anti-CD4 antibodies, and antibody-toxin conjugates; cyclosporine; mycophenolate; mizoribine monophosphate; scoparone; glatiramer acetate; metabolites thereof; pharmaceutically acceptable salts thereof; derivatives thereof; prodrugs thereof; and combinations thereof.

[0070] The term "thiopurine drug" includes azathioprine (AZA), 6-mercaptopurine (6-MP), or any metabolite thereof that has therapeutic efficacy and includes, without limitation, 6-thioguanine (6-TG), 6-methylmercaptopurine riboside, 6-thioinosine nucleotides (*e.g.,* 6-thioinosine monophosphate, 6-thioinosine diphosphate, 6-thioinosine triphosphate), 6-thioguanine nucleotides (*e.g.,* 6-thioguanosine monophosphate, 6-thioguanosine diphosphate, 6-thioguanosine triphosphate), 6-thioxanthosine nucleotides (*e.g.,* 6-thioxanthosine monophosphate, 6-thioxanthosine diphosphate, 6-thioxanthosine triphosphate), derivatives thereof, analogues thereof, and combinations thereof.

[0071] The term "sample" includes any biological specimen obtained from an individual. Samples include, without limitation, whole blood, plasma, serum, red blood cells, white blood cells (*e.g.,* peripheral blood mononuclear cells (PBMC), polymorphonuclear (PMN) cells), ductal lavage fluid, nipple aspirate, lymph (*e.g.,* disseminated tumor cells of the lymph node), bone marrow aspirate, saliva, urine, stool (*i.e.,* feces), sputum, bronchial lavage fluid, tears, fine needle aspirate (*e.g.,* harvested by random periareolar fine needle aspiration), any other bodily fluid, a tissue sample such as a biopsy of a site of inflammation (*e.g.,* needle biopsy), cellular extracts thereof, and an immunoglobulin enriched fraction derived from one or more of these bodily fluids or tissues. ~~In some embodiments, t~~The sample ~~is~~ can be whole blood, a fractional component thereof such as plasma, serum, or a cell pellet, or an immunoglobulin enriched fraction thereof . One skilled in the art will appreciate that samples such as serum samples can be diluted prior to the analysis. ~~In certain embodiments, t~~The sample ~~is~~ can be obtained by isolating PBMCs and/or PMN cells using any technique known in the art. ~~In certain other embodiments, t~~The sample ~~is~~ can be a tissue biopsy such as, *e.g.,* from a site of inflammation such as a portion of the gastrointestinal tract or synovial tissue.

[0072] The steps of the methods ~~of the present invention~~ do not necessarily have to be performed in the particular order in which they are presented. A person of ordinary skill in the art would understand that other orderings of the steps of the methods ~~of the invention~~ are encompassed ~~within the scope of the present invention~~.

[0073] Brackets, "[ ]" indicate that the species within the brackets are referred to by their concentration.

III. Description ~~of the Embodiment~~s

[0074] ~~The present invention provides~~Disclosed assays for detecting and measuring the presence or level of autoantibodies to anti-TNFα drug therapeutics in a sample. The present method~~invention~~ is useful for optimizing therapy and monitoring patients receiving anti-TNFα drug therapeutics to detect the presence or level of autoantibodies (*e.g.,* HACA and/or HAHA) against the drug. ~~The present invention also provides~~Disclosed are methods for selecting therapy, optimizing therapy, and/or reducing toxicity in subjects receiving anti-TNFα drugs for the treatment of TNFα-mediated disease or disorders.

[0075] ~~In one aspect, the present invention provides~~Disclosed is a method for detecting the presence or level of an autoantibody to an anti-TNFα drug in a sample without interference from the anti-TNFα drug in the sample, the method comprising:

(a) contacting the sample with an acid to dissociate preformed complexes of the autoantibody and the anti-TNFα drug, wherein the sample has or is suspected of having an autoantibody to the anti-TNFα drug;

(b) contacting the sample with a labeled anti-TNFα drug following dissociation of the preformed complexes;

(c) neutralizing the acid in the sample to form labeled complexes (*i.e.,* immuno-complexes or conjugates) of the labeled anti-TNFα drug and the autoantibody (*i.e.,* wherein the labeled anti-TNFα drug and autoantibody are not covalently attached to each other);

(d) subjecting the labeled complexes to size exclusion chromatography to separate the labeled complexes (*e.g.,* from free labeled anti-TNFα drug); and

(e) detecting the labeled complexes, thereby detecting the presence or level of the autoantibody without interference from the anti-TNFα drug in the sample.

[0076] Without being bound by any particular theory, it is believed that acid dissociation changes the K_d between the autoantibody (also known as an anti-drug antibody or ADA) and the anti-TNFα drug. In particular, it is theorized that acid dissociation disrupts the bonds between the ADA and the anti-TNFα drug. These bonds include, but are not limited to, hydrogen bonds, electrostatic bonds, Van der Waals forces, and/or hydrophobic bonds. The addition of acid increases the pH and thus the hydrogen ion concentration increases. The hydrogen ions can now compete for the previously mentioned non-covalent interactions. This competition lowers the K_d between the ADA and the anti-TNFα drug.

[0077] ~~In some embodiments,~~Disclosed is that the anti-TNFα drug is selected from the group consisting of REMICADE™ (infliximab), ENBREL™ (etanercept), HUMIRA™ (adalimumab), CIMZIA® (certolizumab pegol), SIMPONI® (golimumab; CNTO 148), and combinations thereof.

[0078] Disclosed is that~~In other embodiments,~~ the anti-TNFα drug autoantibody includes, but is not limited to, human anti-chimeric antibodies (HACA), human anti-humanized antibodies (HAHA), and human anti-mouse antibodies (HAMA), as well as combinations thereof.

[0079] Disclosed is that~~In certain alternative embodiments,~~ steps (a) and (b) are performed simultaneously, *e.g.,* the sample is contacted with an acid and a labeled anti-TNFα drug at the same time. ~~In certain other alternative embodiments, s~~Step (b) ~~is~~ can be performed prior to step (a), *e.g.,* the sample is first contacted with a labeled anti-TNFα drug, and then contacted

with an acid. ~~In further embodiments, s~~Steps (b) and (c) ~~are~~ can be performed simultaneously, *e.g.,* the sample is contacted

with a labeled anti-TNFα drug and neutralized (*e.g.,* by contacting the sample with one or more neutralizing agents) at the same time.

[0080] ~~In particular embodiments, t~~The sample ~~is~~ can be contacted with an amount of an acid that is sufficient to dissociate preformed complexes of the autoantibody and the anti-TNFα drug, such that the labeled anti-TNFα drug, the unlabeled anti-TNFα drug, and the autoantibody to the anti-TNFα drug can equilibrate and form complexes therebetween.

[0081] ~~In preferred embodiments, t~~The methods ~~of the~~can ~~invention~~ comprise detecting the presence or level of the autoantibody without substantial interference from the anti-TNFα drug that is also present in the sample. ~~In such embodiments, t~~The sample can be contacted with an amount of an acid that is sufficient to allow for the detection and/or measurement of the autoantibody in the presence of a high level of the anti-TNFα drug.

[0082] Disclosed is that~~In some embodiments,~~ the phrase "high level of an anti-TNFα drug" can include~~s~~ drug levels of from about 10 to about 100 μg/mL, about 20 to about 80 μg/mL, about 30 to about 70 μg/mL, or about 40 to about 80 μg/mL. ~~In other embodiments, t~~The phrase "high level of an anti-TNFα drug" can include~~s~~ drug levels greater than or equal to about 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100 μg/mL.

[0083] ~~In some embodiments, t~~The acid can comprise~~s~~ an organic acid. ~~In other embodiments, t~~The acid can comprise~~s~~ an inorganic acid. ~~In further embodiments, t~~The acid can comprise~~s~~ a mixture of an organic acid and an inorganic acid. Non-limiting examples of organic acids include citric acid, isocitric acid, glutamic acid, acetic acid, lactic acid, formic acid, oxalic acid, uric acid, trifluoroacetic acid, benzene sulfonic acid, aminomethanesulfonic acid, camphor-10-sulfonic acid, chloroacetic acid, bromoacetic acid, iodoacetic acid, propanoic acid, butanoic acid, glyceric acid, succinic acid, malic acid, aspartic acid, and combinations thereof. Non-limiting examples of inorganic acids include hydrochloric acid, nitric acid, phosphoric acid, sulfuric acid, boric acid, hydrofluoric acid, hydrobromic acid, and combinations thereof.

[0084] ~~In certain embodiments, t~~The amount of an acid can corresponds to a concentration of from about 0.01M to about 10M, about 0.1M to about 5M, about 0.1M to about 2M, about 0.2M to about 1M, or about 0.25M to about 0.75M of an acid or a mixture of acids. ~~In other embodiments, t~~The amount of an acid can corresponds to a concentration of greater than or equal to about 0.01M, 0.05M, 0.1M, 0.2M, 0.3M, 0.4M, 0.5M, 0.6M, 0.7M, 0.8M, 0.9M, 1M, 2M, 3M, 4M, 5M, 6M, 7M, 8M, 9M, or 10M of an acid or a mixture of acids. The pH of the

acid can be, for example, about 0.1, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, or 6.5.

[0085] ~~In some embodiments, t~~The sample ~~is~~ can be contacted with an acid an amount of time that is sufficient to dissociate preformed complexes of the autoantibody and the anti-TNFα drug. In certain instances, the sample is contacted (*e.g.,* incubated) with an acid for a period of time ranging from about 0.1 hours to about 24 hours, about 0.2 hours to about 16 hours, about 0.5 hours to about 10 hours, about 0.5 hours to about 5 hours, or about 0.5 hours to about 2 hours. In other instances, the sample is contacted (*e.g.,* incubated) with an acid for a period of time that is greater than or equal to about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 7, 8, 9, or 10 hours. The sample can be contacted with an acid at 4°C, room temperature (RT), or 37°C.

[0086] ~~In certain embodiments, t~~The step of neutralizing the acid can comprise~~s~~ raising the pH of the sample to allow the formation of complexes between the labeled anti-TNFα drug and the autoantibody to the anti-TNFα drug as well as complexes between unlabeled anti-TNFα drug and the autoantibody. ~~In some embodiments, t~~The acid ~~is~~ can be neutralized by the addition of one or more neutralizing agents such as, for example, strong bases, weak bases, buffer solutions, and combinations thereof. One skilled in the art will appreciate that neutralization reactions do not necessarily imply a resultant pH of 7. In some instances, acid neutralization results in a sample that is basic. In other instances, acid neutralization results in a sample that is acidic (but higher than the pH of the sample prior to adding the neutralizing agent). ~~In particular embodiments, t~~The neutralizing agent can comprise~~s~~ a buffer such as phosphate buffered saline (*e.g.,* 10x PBS) at a pH of about 7.3.

[0087] ~~In some embodiments, s~~Step (b) can further comprise~~s~~ contacting an internal control with the sample together with a labeled anti-TNFα drug (*e.g.,* before, during, or after dissociation of the preformed complexes). In certain instances, the internal control comprises a labeled internal control such as, *e.g.,* Biocytin-Alexa 488. In certain other instances, the amount of the labeled internal control ranges from about 1 ng to about 25 ng, about 5 ng to about 25 ng, about 5 ng to about 20 ng, about 1 ng to about 20 ng, about 1 ng to about 10 ng, or about 1 ng to about 5 ng per 100 µL of sample analyzed. In further instances, the amount of the labeled internal control is greater than or equal to about 1 ng, 5 ng, 10 ng, 15 ng, 20 ng, or 25 ng per 100 µL of sample analyzed.

[0088] As one non-limiting example of the methods ~~of the present invention~~, samples such as serum samples (*e.g.,* serum from subjects receiving therapy with an anti-TNFα drug such as Remicade (IFX)) can be incubated with 0.5M citric acid, pH 3.0 for one hour at room

temperature. Following the dissociation of preformed complexes between (unlabeled) anti-TNFα drug and autoantibodies to the anti-TNFα drug (*e.g.,* anti-drug antibodies such as anti-

IFX antibodies (ATI)), labeled anti-TNFα drug (*e.g.,* IFX-Alexa 488) and an internal control can be added and the reaction mixture and (*e.g.,* immediately) neutralized with a neutralizing agens such as 10x PBS, pH 7.3. After neutralization, the reaction mixture can be incubated for another hour at room temperature (*e.g.,* on a plate shaker) to allow equilibration and to complete the reformation of immune complexes between either the labeled or unlabeled anti-TNFα drug and the anti-drug antibody. The samples can then be filtered and analyzed by SEC-HPLC as described herein.

[0089] ~~In particular embodiments, t~~The methods ~~of the present invention~~ (*e.g.,* comprising acid dissociation followed by homogeneous solution phase binding kinetics) can significantly increase~~s~~ the IFX drug tolerance such that the ATI can be measured in the presence of IFX up to about 60 μg/mL. *See,* Example 14 and Figures 27-28. In other words, the methods ~~of the invention~~ can detect the presence or level of autoantibodies to anti-TNFα drugs such as ATI as well as autoantibodies to other anti-TNFα drugs in the presence of high levels of anti-TNFα drugs (*e.g.,* IFX), but without substantial interference therefrom.

[0090] Disclosed is ~~In another aspect, the present invention provides~~ a method for optimizing therapy and/or reducing toxicity to an anti-TNFα drug in a subject receiving a course of therapy with the anti-TNFα drug, the method comprising:

(a) detecting the presence or level of an autoantibody to the anti-TNFα drug in a sample from the subject without interference from the anti-TNFα drug in the sample, the method comprising:

(i) contacting the sample with an acid to dissociate preformed complexes of the autoantibody and the anti-TNFα drug, wherein the sample has or is suspected of having an autoantibody to the anti-TNFα drug;

(ii) contacting the sample with a labeled anti-TNFα drug following dissociation of the preformed complexes;

(iii) neutralizing the acid in the sample to form labeled complexes (*i.e.,* immuno-complexes or conjugates) of the labeled anti-TNFα drug and the autoantibody (*i.e.,* wherein the labeled anti-TNFα drug and autoantibody are not covalently attached to each other);

(iv) subjecting the labeled complexes to size exclusion chromatography to separate the labeled complexes (*e.g.,* from free labeled anti-TNFα drug); and

(v) detecting the labeled complexes (*e.g.,* thereby detecting the presence or level of the autoantibody without interference from the anti-TNFα drug in the sample); and

(b) determining a subsequent dose of the course of therapy for the subject or whether a different course of therapy should be administered to the subject based upon the presence or level of the autoantibody,

thereby optimizing therapy and/or reducing toxicity to the anti-TNFα drug.

[0091] ~~In certain embodiments, t~~The subsequent dose of the course of therapy ~~is~~ can be increased, decreased, or maintained based upon the presence or level of the autoantibody. As a non-limiting example, a subsequent dose of the course of therapy is decreased when a high level of the autoantibody is detected in the sample. ~~In other embodiments, t~~The different course of therapy can compris~~e~~s a different anti-TNFα drug, the current course of therapy along with an immunosuppressive agent, or switching to a course of therapy that is not an anti-TNFα drug (*e.g.,* discontinuing use of an anti-TNFα therapeutic antibody). As a non-limiting example, a different course of therapy is administered when a high level of the autoantibody is detected in the sample.

[0092] ~~In certain alternative embodiments, s~~Steps (i) and (ii) ~~are~~ can be performed simultaneously, *e.g.,* the sample is contacted with an acid and a labeled anti-TNFα drug at the same time. ~~In certain other alternative embodiments, s~~Step (ii) ~~is~~ can be performed prior to step (i), *e.g.,* the sample is first contacted with a labeled anti-TNFα drug, and then contacted with an acid. ~~In further embodiments, s~~Steps (ii) and (iii) ~~are~~ can be performed simultaneously, *e.g.,* the sample is contacted with a labeled anti-TNFα drug and neutralized (*e.g.,* by contacting the sample with one or more neutralizing agents) at the same time.

[0093] An anti-TNFα drug can be labeled with any of a variety of detectable group(s). ~~In preferred embodiments, a~~An anti-TNFα drug ~~is~~ can be labeled with a fluorophore or a fluorescent dye. Non-limiting examples of fluorophores or fluorescent dyes include those listed in the Molecular Probes Catalogue~~, which is herein incorporated by reference~~ (*see,* R. Haugland, *The Handbook-A Guide to Fluorescent Probes and Labeling Technologies*, 10th Edition, Molecular probes, Inc. (2005)). Such exemplary fluorophores or fluorescent dyes include, but are not limited to, Alexa Fluor® dyes such as Alexa Fluor® 350, Alexa Fluor® 405, Alexa Fluor® 430, Alexa Fluor® 488, Alexa Fluor® 514, Alexa Fluor® 532, Alexa Fluor® 546, Alexa Fluor® 555, Alexa Fluor® 568, Alexa Fluor® 594, Alexa Fluor® 610, Alexa Fluor® 633, Alexa Fluor® 635, Alexa Fluor® 647, Alexa Fluor® 660, Alexa Fluor® 680, Alexa Fluor®

700, Alexa Fluor® 750, and/or Alexa Fluor® 790, as well as other fluorophores including, but not limited

to, Dansyl Chloride (DNS-Cl), 5-(iodoacetamida)fluoroscein (5-IAF), fluoroscein 5-isothiocyanate (FITC), tetramethylrhodamine 5- (and 6-)isothiocyanate (TRITC), 6-acryloyl-2-dimethylaminonaphthalene (acrylodan), 7-nitrobenzo-2-oxa-1,3,-diazol-4-yl chloride (NBD-Cl), ethidium bromide, Lucifer Yellow, 5-carboxyrhodamine 6G hydrochloride, Lissamine rhodamine B sulfonyl chloride, Texas Red™ sulfonyl chloride, BODIPY™, naphthalamine sulfonic acids (*e.g.,* 1-anilinonaphthalene-8-sulfonic acid (ANS), 6-(p-toluidinyl)naphthalen-e-2-sulfonic acid (TNS), and the like), Anthroyl fatty acid, DPH, Parinaric acid, TMA-DPH, Fluorenyl fatty acid, fluorescein-phosphatidylethanolamine, Texas Red-phosphatidylethanolamine, Pyrenyl-phophatidylcholine, Fluorenyl-phosphotidylcholine, Merocyanine 540,1-(3-sulfonatopropyl)-4-[β-[2[(di-n-butylamino)-6 naphthyl]vinyl]pyridinium betaine (Naphtyl Styryl), 3,3'dipropylthiadicarbocyanine (diS-C_3-(5)), 4-(p-dipentyl aminostyryl)-l-methylpyridinium (di-5-ASP), Cy-3 Iodo Acetamide, Cy-5-N-Hydroxysuccinimide, Cy-7-Isothiocyanate, rhodamine 800, IR-125, Thiazole Orange, Azure B, Nile Blue, Al Phthalocyanine, Oxaxine 1, 4', 6-diamidino-2-phenylindole (DAPI), Hoechst 33342, TOTO, Acridine Orange, Ethidium Homodimer, N(ethoxycarbonylmethyl)-6-methoxyquinolinium (MQAE), Fura-2, Calcium Green, Carboxy SNARF-6, BAPTA, coumarin, phytofluors, Coronene, metal-ligand complexes, IRDye® 700DX, IRDye® 700, IRDye® 800RS, IRDye® 800CW, IRDye® 800, Cy5, Cy5.5, Cy7, DY 676, DY680, DY682, DY780, and mixtures thereof. Additional suitable fluorophores include enzyme-cofactors; lanthanide, green fluorescent protein, yellow fluorescent protein, red fluorescent protein, or mutants and derivates thereof. ~~In one embodiment of the invention, t~~The second member of the specific binding pair ~~has~~ can have a detectable group attached thereto.

[0094] Typically, the fluorescent group is a fluorophore selected from the category of dyes comprising polymethines, pthalocyanines, cyanines, xanthenes, fluorenes, rhodamines, coumarins, fluoresceins and BODIPY™.

[0095] ~~In one embodiment, t~~The fluorescent group ~~is~~ can be a near-infrared (NIR) fluorophore that emits in the range of between about 650 to about 900 nm. Use of near infrared fluorescence technology is advantageous in biological assays as it substantially eliminates or reduces background from auto fluorescence of biosubstrates. Another benefit to the near-IR fluorescent technology is that the scattered light from the excitation source is greatly reduced since the scattering intensity is proportional to the inverse fourth power of the wavelength. Low background fluorescence and low scattering result in a high signal to noise ratio, which is essential for highly sensitive detection. Furthermore, the optically transparent window in the near-IR region (650 nm to 900 nm) in biological tissue makes NIR fluorescence a

valuable technology for *in vivo* imaging and subcellular detection applications that require the transmission of light through biological components. ~~Within aspects of this embodiment,~~ ~~t~~The fluorescent group is preferably selected form the group consisting of IRDye® 700DX, IRDye® 700, IRDye® 800RS, IRDye® 800CW, IRDye® 800, Alexa Fluor® 660, Alexa Fluor® 680, Alexa Fluor® 700, Alexa Fluor® 750, Alexa Fluor® 790, Cy5, Cy5.5, Cy7, DY 676, DY680, DY682, and DY780. ~~In certain embodiments, t~~The near infrared group ~~is~~ can be IRDye® 800CW, IRDye® 800, IRDye® 700DX, IRDye® 700, or Dynomic DY676.

[0096] Fluorescent labeling is accomplished using a chemically reactive derivative of a fluorophore. Common reactive groups include amine reactive isothiocyanate derivatives such as FITC and TRITC (derivatives of fluorescein and rhodamine), amine reactive succinimidyl esters such as NHS-fluorescein, and sulfhydryl reactive maleimide activated fluors such as fluorescein-5-maleimide, many of which are commercially available. Reaction of any of these reactive dyes with an anti-TNFα drug results in a stable covalent bond formed between a fluorophore and an anti-TNFα drug.

[0097] In certain instances, following a fluorescent labeling reaction, it is often necessary to remove any nonreacted fluorophore from the labeled target molecule. This is often accomplished by size exclusion chromatography, taking advantage of the size difference between fluorophore and labeled protein.

[0098] Reactive fluorescent dyes are available from many sources. They can be obtained with different reactive groups for attachment to various functional groups within the target molecule. They are also available in labeling kits that contain all the components to carry out a labeling reaction. In one preferred aspect, Alexa Fluor® 647 C2 maleimide is used from Invitrogen (Cat. No. A-20347).

[0099] Specific immunological binding of an anti-drug antibody (ADA) to an anti-TNFα drug can be detected directly or indirectly. Direct labels include fluorescent or luminescent tags, metals, dyes, radionuclides, and the like, attached to the antibody. In certain instances, an anti-TNFα drug that is labeled with iodine-125 (^{125}I) can be used for determining the concentration levels of ADA in a sample. In other instances, a chemiluminescence assay using a chemiluminescent anti-TNFα drug that is specific for ADA in a sample is suitable for sensitive, non-radioactive detection of ADA concentration levels. In particular instances, an anti-TNFα drug that is labeled with a fluorochrome is also suitable for determining the concentration levels of ADA in a sample. Examples of fluorochromes include, without limitation, Alexa Fluor® dyes, DAPI, fluorescein, Hoechst 33258, R-phycocyanin, B-

phycoerythrin, R-phycoerythrin, rhodamine, Texas red, and lissamine. Secondary antibodies linked to fluorochromes can be obtained commercially, *e.g.,* goat F(ab')$_2$ anti-human IgG-FITC is available from Tago Immunologicals (Burlingame, CA).

[0100] Indirect labels include various enzymes well-known in the art, such as horseradish peroxidase (HRP), alkaline phosphatase (AP), β-galactosidase, urease, and the like. A horseradish-peroxidase detection system can be used, for example, with the chromogenic substrate tetramethylbenzidine (TMB), which yields a soluble product in the presence of hydrogen peroxide that is detectable at 450 nm. An alkaline phosphatase detection system can be used with the chromogenic substrate p-nitrophenyl phosphate, for example, which yields a soluble product readily detectable at 405 nm. Similarly, a β-galactosidase detection system can be used with the chromogenic substrate o-nitrophenyl-β-D-galactopyranoside (ONPG), which yields a soluble product detectable at 410 nm. An urease detection system can be used with a substrate such as urea-bromocresol purple (Sigma Immunochemicals; St. Louis, MO). A useful secondary antibody linked to an enzyme can be obtained from a number of commercial sources, *e.g.,* goat F(ab')$_2$ anti-human IgG-alkaline phosphatase can be purchased from Jackson ImmunoResearch (West Grove, PA.).

[0101] A signal from the direct or indirect label can be analyzed, for example, using a spectrophotometer to detect color from a chromogenic substrate; a radiation counter to detect radiation such as a gamma counter for detection of ^{125}I; or a fluorometer to detect fluorescence in the presence of light of a certain wavelength. For detection of enzyme-linked antibodies, a quantitative analysis of ADA levels can be made using a spectrophotometer such as an EMAX Microplate Reader (Molecular Devices; Menlo Park, CA) in accordance with the manufacturer's instructions. If desired, the assays ~~of the present invention~~ can be automated or performed robotically, and the signal from multiple samples can be detected simultaneously.

[0102] ~~In certain embodiments, s~~Size exclusion chromatography ~~is~~ can be used. The underlying principle of SEC is that particles of different sizes will elute (filter) through a stationary phase at different rates. This results in the separation of a solution of particles based on size. Provided that all the particles are loaded simultaneously or near simultaneously, particles of the same size elute together. Each size exclusion column has a range of molecular weights that can be separated. The exclusion limit defines the molecular weight at the upper end of this range and is where molecules are too large to be trapped in the stationary phase. The permeation limit defines the molecular weight at the lower end of the

range of separation and is where molecules of a small enough size can penetrate into the pores of the stationary phase

completely and all molecules below this molecular mass are so small that they elute as a single band.

[0103] In certain aspects, the eluent is collected in constant volumes, or fractions. The more similar the particles are in size, the more likely they will be in the same fraction and not detected separately. Preferably, the collected fractions are examined by spectroscopic techniques to determine the concentration of the particles eluted. Typically, the spectroscopy detection techniques useful in the present method ~~invention~~ include, but are not limited to, fluorometry, refractive index (RI), and ultraviolet (UV). In certain instances, the elution volume decreases roughly linearly with the logarithm of the molecular hydrodynamic volume (*i.e.*, heaver moieties come off first).

[0104] ~~The present invention further provides~~Disclosede is a kit for detecting the presence or level of an autoantibody to an anti-TNFα drug in a sample. ~~In particular embodiments, t~~The kit can comprise~~s~~ one or more of the following components: an acid (or mixture of acids), a labeled anti-TNFα drug (*e.g.,* a labeled anti-TNFα antibody), a labeled internal control, a neutralizing agent (or mixtures thereof), means for detection (*e.g.,* a fluorescence detector), a size exclusion-high performance liquid chromatography (SE-HPLC) instrument, and/or instructions for using the kit.

[0105] ~~In other aspects, the present invention provides~~Disclosed is also a method for selecting a course of therapy (*e.g.,* selecting an appropriate anti-TNFα drug) for the treatment of a TNFα-mediated disease or disorder in a subject, the method comprising:

(a) analyzing a sample obtained from the subject to determine the presence, level, or genotype of one or more markers in the sample;

(b) applying a statistical algorithm to the presence, level, or genotype of the one or more markers determined in step (a) to generate a disease activity/severity index; and

(c) selecting an appropriate course of therapy (*e.g.,* anti-TNFα therapy) for the subject based upon the disease activity/severity index.

[0106] ~~In a related aspect, the present invention provides a~~A method is disclosed for optimizing therapy and/or reducing toxicity in a subject receiving a course of therapy for the treatment of a TNFα-mediated disease or disorder, the method comprising:

(a) analyzing a sample obtained from the subject to determine the presence, level, or genotype of one or more markers in the sample;

(b) applying a statistical algorithm to the presence, level, or genotype of the one or more markers determined in step (a) to generate a disease activity/severity index; and

(c) determining a subsequent dose of the course of therapy for the subject or whether a different course of therapy should be administered to the subject based upon the disease activity/severity index.

[0107] ~~In some embodiments, t~~The course of therapy can comprise~~s~~ an anti-TNFα antibody. In certain instances, the anti-TNFα antibody is a member selected from the group consisting of REMICADE™ (infliximab), ENBREL™ (etanercept), HUMIRA™ (adalimumab), CIMZIA® (certolizumab pegol), SIMPONI® (golimumab; CNTO 148), and combinations thereof. ~~In other embodiments, t~~The course of therapy can comprise~~s~~ an anti-TNFα antibody along with an immunosuppressive agent.

[0108] ~~In certain embodiments, t~~The level of one or more markers can comprise~~s~~ a total level, an activation level, or combinations thereof. In particular instances, the one or more markers is a member selected from the group consisting of an inflammatory marker, a growth factor, a serology marker, a cytokine and/or chemokine, a marker of oxidative stress, a cell surface receptor, a signaling pathway marker, a genetic marker, an anti-TNFα antibody, an anti-drug antibody (ADA), and combinations thereof.

[0109] In some instances, the inflammatory marker is a member selected from the group consisting of CRP, SAA, VCAM, ICAM, calprotectin, lactoferrin, IL-8, Rantes, TNFα, IL-6, IL-1 β, S100A12, M2-pyruvate kinase (PK), IFN, IL-2, TGF, IL-13, IL-15, IL-12, and combinations thereof. In other instances, the growth factor is a member selected from the group consisting of GM-CSF, VEGF, EGF, keratinocyte growth factor (KGF; FGF7), and combinations thereof. In yet other instances, the serology marker is a member selected from the group consisting of an anti-neutrophil antibody, an anti-microbial antibody, an anti-*Saccharomyces cerevisiae* antibody, and combinations thereof. In further instances, the cytokine is a member selected from the group consisting of TNFα, IL-6, IL-1β, IFN-γ, IL-10, and combinations thereof. In other instances, the cell surface receptor is CD64. In yet other instances, the signaling pathway marker is a signal transduction molecule. In other instances, the genetic marker is a mutation in an inflammatory pathway gene.

[0110] ~~In certain embodiments, s~~Step (a) can comprise~~s~~ determining the presence, level, and/or genotype of at least two, three, four, five, six, seven, eight, nine, ten, fifteen, twenty, thirty, forty, fifty, or more markers in the sample. In certain instances, the sample is selected from the group consisting of serum, plasma, whole blood, stool, peripheral blood mononuclear cells (PBMC), polymorphonuclear (PMN) cells, and a tissue biopsy.

[0111] ~~In other embodiments, t~~The statistical algorithm can comprise~~s~~ a learning statistical classifier system. In some instances, the learning statistical classifier system is selected from the group consisting of a random forest, classification and regression tree, boosted tree, neural network, support vector machine, general chi-squared automatic interaction detector model, interactive tree, multiadaptive regression spline, machine learning classifier, and combinations thereof. In certain instances, the statistical algorithm comprises a single learning statistical classifier system. In certain other instances, the statistical algorithm comprises a combination of at least two learning statistical classifier systems. In some instances, the at least two learning statistical classifier systems are applied in tandem. Non-limiting examples of statistical algorithms and analysis suitable for use in the method ~~invention~~ are described in International Application No. PCT/US2011/056777, filed October 18, 2011~~, the disclosure of which is hereby incorporated by reference in its entirety for all purposes~~.

[0112] ~~In some embodiments, t~~The method further can comprise~~s~~ sending the results from the selection or determination of step (c) to a clinician. ~~In other embodiments, s~~Step (c) can comprise~~s~~ selecting an initial course of therapy for the subject.

[0113] ~~In other embodiments, s~~Step (b) can further comprise~~s~~ applying a statistical algorithm to the presence, level, or genotype of one or more markers determined at an earlier time during the course of therapy to generate an earlier disease activity/severity index. In some instances, the earlier disease activity/severity index is compared to the disease activity/severity index generated in step (b) to determine a subsequent dose of the course of therapy or whether a different course of therapy should be administered. ~~In certain embodiments, t~~The subsequent dose of the course of therapy ~~is~~ can be increased, decreased, or maintained based upon the disease activity/severity index generated in step (b). In some instances, the different course of therapy comprises a different anti-TNFα antibody. In other instances, the different course of therapy comprises the current course of therapy along with an immunosuppressive agent.

[0114] Methods for detecting anti-TNFα antibodies and anti-drug antibodies (ADA) are described herein and in PCT Publication No. WO 2011/056590~~, the disclosure of which is hereby incorporated by reference in its entirety for all purposes~~. In particular ~~embodiments~~, the presence or level of anti-drug antibodies ~~is~~ can be determined in accordance with the methods ~~of the invention~~ comprising an acid dissociation step by contacting a sample with an acid prior to, during, and/or after contacting the sample with a labeled anti-TNFα drug.

[0115] ~~In another aspect, the present invention provides~~Disclosed is also a method for predicting the course of a TNFα-mediated disease or disorder in a subject, the method comprising:

(a) analyzing a sample obtained from the subject to determine the presence, level, or genotype of one or more markers in the sample;

(b) applying a statistical algorithm to the presence, level, or genotype of the one or more markers determined in step (a) to generate a disease activity/severity index; and

(c) predicting the course of the TNFα-mediated disease or disorder based upon the disease activity/severity index generated in step (b).

[0116] ~~In some embodiments, s~~Step (b) can further comprise~~s~~ applying a statistical algorithm to the presence, level, or genotype of one or more of the markers determined at an earlier time to generate an earlier disease activity/severity index. In certain instances, the earlier disease activity/severity index is compared to the disease activity/severity index generated in step (b) to predict the course of the TNFα-mediated disease or disorder.

[0117] Once the diagnosis or prognosis of a subject receiving anti-TNFα drug therapy has been determined or the likelihood of response to an anti-TNFα drug has been predicted in a subject diagnosed with a disease and disorder in which TNFα has been implicated in the pathophysiology, *e.g.*, but not limited to, shock, sepsis, infections, autoimmune diseases, RA, Crohn's disease, transplant rejection and graft-versus-host disease, according to the methods described herein, the present ~~invention~~ method may further comprise recommending a course of therapy based upon the diagnosis, prognosis, or prediction. ~~In certain instances, the present invention~~ Disclosed is that the method may further comprise administering to a subject a therapeutically effective amount of an anti-TNFα drug useful for treating one or more symptoms associated with the TNFα-mediated disease or disorder. For therapeutic applications, the anti-TNFα drug can be administered alone or co-administered in combination with one or more additional anti-TNFα drugs and/or one or more drugs that reduce the side-effects associated with the anti-TNFα drug (*e.g.,* an immunosuppressive agent). As such, the present method ~~invention~~ advantageously enables a clinician to practice "personalized medicine" by guiding treatment decisions and informing therapy selection and optimization for anti-TNFα drugs such that the right drug is given to the right patient at the right time.

IV. Disease Activity/Severity Index

[0118] In certain aspects, the present method ~~invention~~ provides an algorithmic-based analysis of one or a plurality of (*e.g.,* two, three, four, five, six, seven, or more) biomarkers to improve the accuracy of selecting therapy, optimizing therapy, reducing toxicity, and/or monitoring the efficacy of therapeutic treatment to anti-TNFα drug therapy.

[0119] As a non-limiting example, the disease activity/severity index ~~in one embodiment~~ can comprise~~s~~ detecting, measuring, or determining the presence, level (concentration (*e.g.,* total) and/or activation (*e.g.,* phosphorylation)), or genotype of one or more specific biomarkers in one or more of the following categories of biomarkers:

(1) Inflammatory markers

(2) Growth factors

(3) Serology (*e.g.,* immune markers)

(4) Cytokines and chemokines

(5) Markers of oxidative stress

(6) Cell surface receptors (*e.g.,* CD64, others)

(7) Signaling pathways

(8) Other markers (*e.g.,* genetic markers such as inflammatory pathway genes).

[0120] ~~In further embodiments, t~~The presence and/or level of one or both of the following markers can also be detected, measured, or determined in a patient sample (*e.g.,* a serum sample from a patient on anti-TNFα drug therapy): (9) anti-TNFα drug levels (*e.g.,* levels of free anti-TNFα therapeutic antibody); and/or (10) anti-drug antibody (ADA) levels (*e.g.,* levels of autoantibody to the anti-TNFα drug).

[0121] A single statistical algorithm or a combination of two or more statistical algorithms described herein can then be applied to the presence, concentration level, activation level, or genotype of the markers detected, measured, or determined in the sample to thereby select therapy, optimize therapy, reduce toxicity, or monitor the efficacy of therapeutic treatment with an anti-TNFα drug. As such, the methods ~~of the invention~~ find utility in determining patient management by determining patient immune status.

[0122] Understanding the clinical course of disease will enable physicians to make better informed treatment decisions for their inflammatory disease patients(*e.g.,* IBD (*e.g.,* Crohn's disease), rheumatoid arthritis (RA), others) and may help to direct new drug development in the future. The ideal biomarker(s) for use in the disease activity/severity index described herein should be able to identify individuals at risk for the disease and should be disease-specific. Moreover, the biomarker(s) should be able to detect disease activity and monitor the effect of treatment; and should have a predictive value towards relapse or recurrence of the disease. Predicting disease course, however, has now been expanded beyond just disease recurrence, but perhaps more importantly to include predictors of disease complications including surgery. The present ~~invention~~ method is particularly advantageous because it

provides indicators of disease activity and/or severity and enables a prediction of the risk of relapse in

those patients in remission. In addition, the biomarkers and disease activity/severity index of present ~~invention~~ method have enormous implications for patient management as well as therapeutic decision-making and would aid or assist in directing the appropriate therapy to those patients who would most likely benefit from it and avoid the expense and potential toxicity of chronic maintenance therapy in those who have a low risk of recurrence.

A. Inflammatory Markers

[0123] Although disease course of an inflammatory disease is typically measured in terms of inflammatory activity by noninvasive tests using white blood cell count, this method has a low specificity and shows limited correlation with disease activity.

[0124] As such, ~~in certain embodiments,~~ a variety of inflammatory markers, including biochemical markers, serological markers, protein markers, genetic markers, and other clinical or echographic characteristics, are particularly useful in the methods ~~of the present invention~~ for selecting therapy, optimizing therapy, reducing toxicity, and/or monitoring the efficacy of therapeutic treatment with one or more therapeutic agents such as biologics (*e.g.,* anti-TNFα drugs). In certain aspects, the methods described herein utilize the application of an algorithm (*e.g.,* statistical analysis) to the presence, concentration level, and/or genotype determined for one or more inflammatory markers (*e.g.,* alone or in combination with biomarkers from other categories) to aid or assist in predicting disease course, selecting an appropriate anti-TNFα drug therapy, optimizing anti-TNFα drug therapy, reducing toxicity associated with anti-TNFα drug therapy, or monitoring the efficacy of therapeutic treatment with an anti-TNFα drug.

[0125] Non-limiting examples of inflammatory markers suitable for use in the present ~~invention~~ method include biochemical, serological, and protein markers such as, *e.g.,* cytokines, chemokines, acute phase proteins, cellular adhesion molecules, S100 proteins, and/or other inflammatory markers.

1. Cytokines and Chemokines

[0126] The determination of the presence or level of at least one cytokine or chemokine in a sample is particularly useful in the present method ~~invention~~. As used herein, the term "cytokine" includes any of a variety of polypeptides or proteins secreted by immune cells that regulate a range of immune system functions and encompasses small cytokines such as chemokines. The term "cytokine" also includes adipocytokines, which comprise a group of cytokines secreted by adipocytes that function, for example, in the regulation of body weight,

[0129] The human IL-6 polypeptide sequence is set forth in, *e.g.*, Genbank Accession No. NP_000591. The human IL-6 mRNA (coding) sequence is set forth in, *e.g.*, Genbank Accession No. NM_000600. One skilled in the art will appreciate that IL-6 is also known as interferon beta 2 (IFNB2), HGF, HSF, and BSF2.

[0130] The human IL-1β polypeptide sequence is set forth in, *e.g.*, Genbank Accession No. NP_000567. The human IL-1β mRNA (coding) sequence is set forth in, *e.g.*, Genbank Accession No. NM_000576. One skilled in the art will appreciate that IL-1β is also known as IL1F2 and IL-1beta.

[0131] The human IL-8 polypeptide sequence is set forth in, *e.g.*, Genbank Accession No. NP_000575 (SEQ ID NO:1). The human IL-8 mRNA (coding) sequence is set forth in, *e.g.*, Genbank Accession No. NM_000584 (SEQ ID NO:2). One skilled in the art will appreciate that IL-8 is also known as CXCL8, K60, NAF, GCP1, LECT, LUCT, NAP1, 3-10C, GCP-1, LYNAP, MDNCF, MONAP, NAP-1, SCYB8, TSG-1, AMCF-I, and b-ENAP.

[0132] The human TWEAK polypeptide sequence is set forth in, *e.g.*, Genbank Accession Nos. NP_003800 and AAC51923. The human TWEAK mRNA (coding) sequence is set forth in, *e.g.*, Genbank Accession Nos. NM_003809 and BC104420. One skilled in the art will appreciate that TWEAK is also known as tumor necrosis factor ligand superfamily member 12 (TNFSF12), APO3 ligand (APO3L), CD255, DR3 ligand, growth factor-inducible 14 (Fn14) ligand, and UNQ181/PRO207.

2. Acute Phase Proteins

[0133] The determination of the presence or level of one or more acute-phase proteins in a sample is also useful in the present method ~~invention~~. Acute-phase proteins are a class of proteins whose plasma concentrations increase (positive acute-phase proteins) or decrease (negative acute-phase proteins) in response to inflammation. This response is called the acute-phase reaction (also called acute-phase response). Examples of positive acute-phase proteins include, but are not limited to, C-reactive protein (CRP), D-dimer protein, mannose-binding protein, alpha 1-antitrypsin, alpha 1-antichymotrypsin, alpha 2-macroglobulin, fibrinogen, prothrombin, factor VIII, von Willebrand factor, plasminogen, complement factors, ferritin, serum amyloid P component, serum amyloid A (SAA), orosomucoid (alpha 1-acid glycoprotein, AGP), ceruloplasmin, haptoglobin, and combinations thereof. Non-limiting examples of negative acute-phase proteins include albumin, transferrin, transthyretin, transcortin, retinol-binding protein, and combinations thereof. Preferably, the presence or level of CRP and/or SAA is determined.

3. Cellular Adhesion Molecules (IgSF CAMs)

[0137] The determination of the presence or level of one or more immunoglobulin superfamily cellular adhesion molecules in a sample is also useful in the present method ~~invention~~. As used herein, the term "immunoglobulin superfamily cellular adhesion molecule" (IgSF CAM) includes any of a variety of polypeptides or proteins located on the surface of a cell that have one or more immunoglobulin-like fold domains, and which function in intercellular adhesion and/or signal transduction. In many cases, IgSF CAMs are transmembrane proteins. Non-limiting examples of IgSF CAMs include Neural Cell Adhesion Molecules (NCAMs; *e.g.*, NCAM-120, NCAM-125, NCAM-140, NCAM-145, NCAM-180, NCAM-185, *etc.*), Intercellular Adhesion Molecules (ICAMs, *e.g.*, ICAM-1, ICAM-2, ICAM-3, ICAM-4, and ICAM-5), Vascular Cell Adhesion Molecule-1 (VCAM-1), Platelet-Endothelial Cell Adhesion Molecule-1 (PECAM-1), L1 Cell Adhesion Molecule (L1CAM), cell adhesion molecule with homology to L1CAM (close homolog of L1) (CHL1), sialic acid binding Ig-like lectins (SIGLECs; *e.g.*, SIGLEC-1, SIGLEC-2, SIGLEC-3, SIGLEC-4, *etc.*), Nectins (*e.g.*, Nectin-1, Nectin-2, Nectin-3, *etc.*), and Nectin-like molecules (*e.g.*, Necl-1, Necl-2, Necl-3, Necl-4, and Necl-5). Preferably, the presence or level of ICAM-1 and/or VCAM-1 is determined.

[0138] ICAM-1 is a transmembrane cellular adhesion protein that is continuously present in low concentrations in the membranes of leukocytes and endothelial cells. Upon cytokine stimulation, the concentrations greatly increase. ICAM-1 can be induced by IL-1 and TNFα and is expressed by the vascular endothelium, macrophages, and lymphocytes. In IBD, proinflammatory cytokines cause inflammation by upregulating expression of adhesion molecules such as ICAM-1 and VCAM-1. The increased expression of adhesion molecules recruit more lymphocytes to the infected tissue, resulting in tissue inflammation (*see,* Goke *et al*., *J., Gastroenterol.*, 32:480 (1997); and Rijcken *et al*., *Gut*, 51:529 (2002)). ICAM-1 is encoded by the intercellular adhesion molecule 1 gene (ICAM1; Entrez GeneID:3383; Genbank Accession No. NM_000201) and is produced after processing of the intercellular adhesion molecule 1 precursor polypeptide (Genbank Accession No. NP_000192).

[0139] VCAM-1 is a transmembrane cellular adhesion protein that mediates the adhesion of lymphocytes, monocytes, eosinophils, and basophils to vascular endothelium. Upregulation of VCAM-1 in endothelial cells by cytokines occurs as a result of increased gene transcription (*e.g.,* in response to Tumor necrosis factor-alpha (TNFα) and Interleukin-1 (IL-1)). VCAM-1 is encoded by the vascular cell adhesion molecule 1 gene (VCAM1;

Entrez GeneID:7412) and is produced after differential splicing of the transcript (Genbank Accession No. NM_001078 (variant 1) or NM_080682 (variant 2)), and processing of the precursor polypeptide splice isoform (Genbank Accession No. NP_001069 (isoform a) or NP_542413 (isoform b)).

[0140] In certain instances, the presence or level of an IgSF CAM is detected at the level of mRNA expression with an assay such as, for example, a hybridization assay or an amplification-based assay. In certain other instances, the presence or level of an IgSF CAM is detected at the level of protein expression using, for example, an immunoassay (*e.g.,* ELISA) or an immunohistochemical assay. Suitable antibodies and/or ELISA kits for determining the presence or level of ICAM-1 and/or VCAM-1 in a sample such as a tissue sample, biopsy, serum, plasma, saliva, urine, or stool are available from, *e.g.,* Invitrogen (Camarillo, CA), Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and/or Abcam Inc. (Cambridge, MA).

4. S100 Proteins

[0141] The determination of the presence or level of at least one S100 protein in a sample is also useful in the present method ~~invention~~. As used herein, the term "S100 protein" includes any member of a family of low molecular mass acidic proteins characterized by cell-type-specific expression and the presence of 2 EF-hand calcium-binding domains. There are at least 21 different types of S100 proteins in humans. The name is derived from the fact that S100 proteins are 100% soluble in ammonium sulfate at neutral pH. Most S100 proteins are homodimeric, consisting of two identical polypeptides held together by non-covalent bonds. Although S100 proteins are structurally similar to calmodulin, they differ in that they are cell-specific, expressed in particular cells at different levels depending on environmental factors. S-100 proteins are normally present in cells derived from the neural crest (*e.g.,* Schwann cells, melanocytes, glial cells), chondrocytes, adipocytes, myoepithelial cells, macrophages, Langerhans cells, dendritic cells, and keratinocytes. S100 proteins have been implicated in a variety of intracellular and extracellular functions such as the regulation of protein phosphorylation, transcription factors, Ca^{2+} homeostasis, the dynamics of cytoskeleton constituents, enzyme activities, cell growth and differentiation, and the inflammatory response.

[0142] Calgranulin is an S100 protein that is expressed in multiple cell types, including renal epithelial cells and neutrophils, and are abundant in infiltrating monocytes and granulocytes under conditions of chronic inflammation. Examples of calgranulins include,

without limitation, calgranulin A (also known as S100A8 or MRP-8), calgranulin B (also known as S100A9 or MRP-14), and calgranulin C (also known as S100A12).

[0143] In certain instances, the presence or level of a particular S100 protein is detected at the level of mRNA expression with an assay such as, for example, a hybridization assay or an amplification-based assay. In certain other instances, the presence or level of a particular S100 protein is detected at the level of protein expression using, for example, an immunoassay (*e.g.,* ELISA) or an immunohistochemical assay. Suitable ELISA kits for determining the presence or level of an S100 protein such as calgranulin A (S100A8), calgranulin B (S100A9), or calgranulin C (S100A12) in a serum, plasma, or urine sample are available from, *e.g.,* Peninsula Laboratories Inc. (San Carlos, CA) and Hycult biotechnology b.v. (Uden, The Netherlands).

[0144] Calprotectin, the complex of S100A8 and S100A9, is a calcium- and zinc-binding protein in the cytosol of neutrophils, monocytes, and keratinocytes. Calprotectin is a major protein in neutrophilic granulocytes and macrophages and accounts for as much as 60% of the total protein in the cytosol fraction in these cells. It is therefore a surrogate marker of neutrophil turnover. Its concentration in stool correlates with the intensity of neutrophil infiltration of the intestinal mucosa and with the severity of inflammation. In some instances, calprotectin can be measured with an ELISA using small (50-100 mg) fecal samples (*see*, *e.g.,* Johne *et al.*, *Scand J Gastroenterol*., 36:291-296 (2001)).

5. Other Inflammatory Markers

[0145] The determination of the presence or level of lactoferrin in a sample is also useful in the present method ~~invention~~. In certain instances, the presence or level of lactoferrin is detected at the level of mRNA expression with an assay such as, for example, a hybridization assay or an amplification-based assay. In certain other instances, the presence or level of lactoferrin is detected at the level of protein expression using, for example, an immunoassay (*e.g.,* ELISA) or an immunohistochemical assay. A lactoferrin ELISA kit available from Calbiochem (San Diego, CA) can be used to detect human lactoferrin in a plasma, urine, bronchoalveolar lavage, or cerebrospinal fluid sample. Similarly, an ELISA kit available from U.S. Biological (Swampscott, MA) can be used to determine the level of lactoferrin in a plasma sample. U.S. Patent Publication No. 20040137536 describes an ELISA assay for determining the presence of elevated lactoferrin levels in a stool sample. Likewise, U.S. Patent Publication No. 20040033537 describes an ELISA assay for determining the concentration of endogenous lactoferrin in a stool, mucus, or bile sample. ~~In some embodiments, then~~The presence or level of

anti-lactoferrin antibodies can be detected in a sample using, *e.g.,* lactoferrin protein or a fragment thereof.

[0146] The determination of the presence or level of one or more pyruvate kinase isozymes such as M1-PK and M2-PK in a sample is also useful ~~in the present invention~~. In certain instances, the presence or level of M1-PK and/or M2-PK is detected at the level of mRNA expression with an assay such as, for example, a hybridization assay or an amplification-based assay. In certain other instances, the presence or level of M1-PK and/or M2-PK is detected at the level of protein expression using, for example, an immunoassay (*e.g.,* ELISA) or an immunohistochemical assay. Pyruvate kinase isozymes M1/M2 are also known as pyruvate kinase muscle isozyme (PKM), pyruvate kinase type K, cytosolic thyroid hormone-binding protein (CTHBP), thyroid hormone-binding protein 1 (THBP1), or opa-interacting protein 3 (OIP3).

[0147] ~~In further embodiments, t~~The determination of the presence or level of one or more growth factors in a sample ~~is~~ can also useful in the present method ~~invention~~. Non-limiting examples of growth factors include transforming growth factors (TGF) such as TGF-α, TGF-β, TGF-β2, TGF-β3, *etc.*, which are described in detail below.

6. Exemplary Set of Inflammatory Markers

[0148] ~~In particular embodiments, a~~At least one or a plurality (*e.g.,* two, three, four, five, six, seven, eight, nine, ten, or more such as, *e.g.,* a panel) of the following inflammatory markers can be detected (*e.g.,* alone or in combination with biomarkers from other categories) to aid or assist in predicting disease course, and/or to improve the accuracy of selecting therapy, optimizing therapy, reducing toxicity, and/or monitoring the efficacy of therapeutic treatment to anti-TNFα drug therapy:

a. CRP
b. SAA
c. VCAM
d. ICAM
e. Calprotectin
f. Lactoferrin
g. IL8
h. Rantes
i. TNFalpha
j. IL-6

k. IL-1beta

l. S100A12

m. M2-pyruvate kinase (PK)

n. IFN

o. IL2

p. TGF

q. IL-13

r. IL-15

s. IL12

t. Other chemokines and cytokines.

B. Growth Factors

[0149] A variety of growth factors, including biochemical markers, serological markers, protein markers, genetic markers, and other clinical or echographic characteristics, are suitable for use in the methods ~~of the present invention~~ for selecting therapy, optimizing therapy, reducing toxicity, and/or monitoring the efficacy of therapeutic treatment with one or more therapeutic agents such as biologics (*e.g.,* anti-TNFα drugs). In certain aspects, the methods described herein utilize the application of an algorithm (*e.g.,* statistical analysis) to the presence, concentration level, and/or genotype determined for one or more growth factors (*e.g.,* alone or in combination with biomarkers from other categories) to aid or assist in predicting disease course, selecting an appropriate anti-TNFα drug therapy, optimizing anti-TNFα drug therapy, reducing toxicity associated with anti-TNFα drug therapy, or monitoring the efficacy of therapeutic treatment with an anti-TNFα drug.

[0150] As such, ~~in certain embodiments,~~ the determination of the presence or level of one or more growth factors in a sample ~~is~~ can be useful in the present method ~~invention~~. As used herein, the term "growth factor" includes any of a variety of peptides, polypeptides, or proteins that are capable of stimulating cellular proliferation and/or cellular differentiation.

[0151] In certain aspects, the presence or level of at least one growth factor including, but not limited to, epidermal growth factor (EGF), heparin-binding epidermal growth factor (HB-EGF), vascular endothelial growth factor (VEGF), pigment epithelium-derived factor (PEDF; also known as SERPINF1), amphiregulin (AREG; also known as schwannoma-derived growth factor (SDGF)), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), transforming growth factor-α (TGF-α), transforming growth factor-β (TGF-β1, TGF-β2, TGF-β3, *etc.*), endothelin-1 (ET-1), keratinocyte growth factor (KGF; also known as

FGF7), bone morphogenetic proteins (*e.g.,* BMP1-BMP15), platelet-derived growth factor (PDGF), nerve growth factor (NGF), β-nerve growth factor (β-NGF), neurotrophic factors (*e.g.,* brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3), neurotrophin 4 (NT4), *etc.*), growth differentiation factor-9 (GDF-9), granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), myostatin (GDF-8), erythropoietin (EPO), and thrombopoietin (TPO) is determined in a sample. In particular ~~embodiments~~, the presence or level of at least one of VEGF, EGF, bFGF, ET-1, TGF-β2 and/or TGF-β3 ~~is~~ can be determined. These markers have been found to be significantly higher in active IBD than in controls, indicating that they may play a role in promoting healing after mucosal injury of the luminal surface of the intestine in IBD.

[0152] In certain instances, the presence or level of a particular growth factor is detected at the level of mRNA expression with an assay such as, for example, a hybridization assay or an amplification-based assay. In certain other instances, the presence or level of a particular growth factor is detected at the level of protein expression using, for example, an immunoassay (*e.g.,* ELISA) or an immunohistochemical assay. Suitable ELISA kits for determining the presence or level of a growth factor in a serum, plasma, saliva, or urine sample are available from, *e.g.,* Antigenix America Inc. (Huntington Station, NY), Promega (Madison, WI), R&D Systems, Inc. (Minneapolis, MN), Invitrogen (Camarillo, CA), CHEMICON International, Inc. (Temecula, CA), Neogen Corp. (Lexington, KY), PeproTech (Rocky Hill, NJ), Alpco Diagnostics (Salem, NH), Pierce Biotechnology, Inc. (Rockford, IL), and/or Abazyme (Needham, MA).

[0153] The human epidermal growth factor (EGF) polypeptide sequence is set forth in, *e.g.*, Genbank Accession No. NP_001954 (SEQ ID NO:19). The human EGF mRNA (coding) sequence is set forth in, *e.g.*, Genbank Accession No. NM_001963 (SEQ ID NO:20). One skilled in the art will appreciate that EGF is also known as beta-urogastrone, URG, and HOMG4.

[0154] The human vascular endothelial growth factor (VEGF) polypeptide sequence is set forth in, *e.g.*, Genbank Accession Nos. NP_001020537 (SEQ ID NO:21), NP_001020538, NP_001020539, NP_001020540, NP_001020541, NP_001028928, and NP_003367. The human VEGF mRNA (coding) sequence is set forth in, *e.g.*, Genbank Accession No. NM_001025366 (SEQ ID NO:22), NM_001025367, NM_001025368, NM_001025369, NM_001025370, NM_001033756, and NM_003376. One skilled in the art will appreciate that VEGF is also known as VPF, VEGFA, VEGF-A, and MGC70609.

[0155] ~~In particular embodiments, a~~At least one or a plurality (*e.g.,* two, three, four, five, six, seven, eight, nine, ten, or more such as, *e.g.,* a panel) of the following growth factors can be detected (*e.g.,* alone or in combination with biomarkers from other categories) to aid or assist in predicting disease course, and/or to improve the accuracy of selecting therapy, optimizing therapy, reducing toxicity, and/or monitoring the efficacy of therapeutic treatment to anti-TNFα drug therapy: GM-CSF; VEGF; EGF; Keratinocyte growth factor (KGF; FGF7); and other growth factors.

C. Serology (Immune Markers)

[0156] The determination of serological or immune markers such as autoantibodies in a sample (*e.g.,* serum sample) is also useful in the present method ~~invention~~. Antibodies against anti-inflammatory molecules such as IL-10, TGF-β, and others might suppress the body's ability to control inflammation and the presence or level of these antibodies in the patient indicates the use of powerful immunosuppressive medications such as anti-TNFα drugs. Mucosal healing might result in a decrease in the antibody titre of antibodies to bacterial antigens such as, *e.g.,* OmpC, flagellins (cBir-1, Fla-A, Fla-X, *etc.*), I2, and others (pANCA, ASCA, *etc.*).

[0157] As such, in certain aspects, the methods described herein utilize the application of an algorithm (*e.g.,* statistical analysis) to the presence, concentration level, and/or genotype determined for one or more immune markers (*e.g.,* alone or in combination with biomarkers from other categories) to aid or assist in predicting disease course, selecting an appropriate anti-TNFα drug therapy, optimizing anti-TNFα drug therapy, reducing toxicity associated with anti-TNFα drug therapy, or monitoring the efficacy of therapeutic treatment with an anti-TNFα drug.

[0158] Non-limiting examples of serological immune markers suitable for use in the present method ~~invention~~ include anti-neutrophil antibodies, anti-*Saccharomyces cerevisiae* antibodies, and/or other anti-microbial antibodies.

1. Anti-Neutrophil Antibodies

[0159] The determination of ANCA levels and/or the presence or absence of pANCA in a sample is useful in the methods ~~of the present invention~~. As used herein, the term "anti-neutrophil cytoplasmic antibody" or "ANCA" includes antibodies directed to cytoplasmic and/or nuclear components of neutrophils. ANCA activity can be divided into several broad categories based upon the ANCA staining pattern in neutrophils: (1) cytoplasmic neutrophil

staining without perinuclear highlighting (cANCA); (2) perinuclear staining around the outside edge of the nucleus (pANCA); (3) perinuclear staining around the inside edge of the

nucleus (NSNA); and (4) diffuse staining with speckling across the entire neutrophil (SAPPA). In certain instances, pANCA staining is sensitive to DNase treatment. The term ANCA encompasses all varieties of anti-neutrophil reactivity, including, but not limited to, cANCA, pANCA, NSNA, and SAPPA. Similarly, the term ANCA encompasses all immunoglobulin isotypes including, without limitation, immunoglobulin A and G.

[0160] ANCA levels in a sample from an individual can be determined, for example, using an immunoassay such as an enzyme-linked immunosorbent assay (ELISA) with alcohol-fixed neutrophils. The presence or absence of a particular category of ANCA such as pANCA can be determined, for example, using an immunohistochemical assay such as an indirect fluorescent antibody (IFA) assay. Preferably, the presence or absence of pANCA in a sample is determined using an immunofluorescence assay with DNase-treated, fixed neutrophils. In addition to fixed neutrophils, antigens specific for ANCA that are suitable for determining ANCA levels include, without limitation, unpurified or partially purified neutrophil extracts; purified proteins, protein fragments, or synthetic peptides such as histone H1 or ANCA-reactive fragments thereof (*see, e.g.,* U.S. Patent No. 6,074,835); histone H1-like antigens, porin antigens, Bacteroides antigens, or ANCA-reactive fragments thereof (*see, e.g.,* U.S. Patent No. 6,033,864); secretory vesicle antigens or ANCA-reactive fragments thereof (*see, e.g.,* U.S. Patent Application No. 08/804,106); and anti-ANCA idiotypic antibodies. One skilled in the art will appreciate that the use of additional antigens specific for ANCA is possible with the present method~~within the scope of the present invention~~.

2. Anti-*Saccharomyces cerevisiae* Antibodies

[0161] The determination of ASCA (*e.g.,* ASCA-IgA and/or ASCA-IgG) levels in a sample is useful in the present method ~~invention~~. As used herein, the term "anti-*Saccharomyces cerevisiae* immunoglobulin A" or "ASCA-IgA" includes antibodies of the immunoglobulin A isotype that react specifically with *S. cerevisiae*. Similarly, the term "anti-*Saccharomyces cerevisiae* immunoglobulin G" or "ASCA-IgG" includes antibodies of the immunoglobulin G isotype that react specifically with *S. cerevisiae*.

[0162] The determination of whether a sample is positive for ASCA-IgA or ASCA-IgG is made using an antigen specific for ASCA. Such an antigen can be any antigen or mixture of antigens that is bound specifically by ASCA-IgA and/or ASCA-IgG. Although ASCA antibodies were initially characterized by their ability to bind *S. cerevisiae*, those of skill in the art will understand that an antigen that is bound specifically by ASCA can be obtained from *S. cerevisiae* or from a variety of other sources so long as the antigen is capable of

binding specifically to ASCA antibodies. Accordingly, exemplary sources of an antigen specific for ASCA, which can be used to determine the levels of ASCA-IgA and/or ASCA-IgG in a sample, include, without limitation, whole killed yeast cells such as *Saccharomyces* or *Candida* cells; yeast cell wall mannan such as phosphopeptidomannan (PPM); oligosachharides such as oligomannosides; neoglycolipids; anti-ASCA idiotypic antibodies; and the like. Different species and strains of yeast, such as *S. cerevisiae* strain Su1, Su2, CBS 1315, or BM 156, or *Candida albicans* strain VW32, are suitable for use as an antigen specific for ASCA-IgA and/or ASCA-IgG. Purified and synthetic antigens specific for ASCA are also suitable for use in determining the levels of ASCA-IgA and/or ASCA-IgG in a sample. Examples of purified antigens include, without limitation, purified oligosaccharide antigens such as oligomannosides. Examples of synthetic antigens include, without limitation, synthetic oligomannosides such as those described in U.S. Patent Publication No. 20030105060, *e.g.,* D-Man β(1-2) D-Man β(1-2) D-Man β(1-2) D-Man-OR, D-Man α(1-2) D-Man α(1-2) D-Man α(1-2) D-Man-OR, and D-Man α(1-3) D-Man α(1-2) D-Man α(1-2) D-Man-OR, wherein R is a hydrogen atom, a C_1 to C_{20} alkyl, or an optionally labeled connector group.

[0163] Preparations of yeast cell wall mannans, *e.g.,* PPM, can be used in determining the levels of ASCA-IgA and/or ASCA-IgG in a sample. Such water-soluble surface antigens can be prepared by any appropriate extraction technique known in the art, including, for example, by autoclaving, or can be obtained commercially (*see, e.g.,* Lindberg *et al.*, *Gut*, 33:909-913 (1992)). The acid-stable fraction of PPM is also useful in the statistical algorithms ~~of the present invention~~ (Sendid *et al.*, *Clin. Diag. Lab. Immunol.*, 3:219-226 (1996)). An exemplary PPM that is useful in determining ASCA levels in a sample is derived from *S. uvarum* strain ATCC #38926.

[0164] Purified oligosaccharide antigens such as oligomannosides can also be useful in determining the levels of ASCA-IgA and/or ASCA-IgG in a sample. The purified oligomannoside antigens are preferably converted into neoglycolipids as described in, for example, Faille *et al.*, *Eur. J. Microbiol. Infect. Dis.*, 11:438-446 (1992). One skilled in the art understands that the reactivity of such an oligomannoside antigen with ASCA can be optimized by varying the mannosyl chain length (Frosh *et al.*, *Proc Natl. Acad. Sci. USA*, 82:1194-1198 (1985)); the anomeric configuration (Fukazawa *et al.*, In "Immunology of Fungal Disease," E. Kurstak (ed.), Marcel Dekker Inc., New York, pp. 37-62 (1989); Nishikawa *et al.*, *Microbiol. Immunol.*, 34:825-840 (1990); Poulain *et al.*, *Eur. J. Clin. Microbiol.*, 23:46-52 (1993); Shibata *et al.*, *Arch. Biochem. Biophys.*, 243:338-348 (1985);

Trinel *et al.*, *Infect. Immun.*, 60:3845-3851 (1992)); or the position of the linkage (Kikuchi *et al.*, *Planta*, 190:525-535 (1993)).

[0165] Suitable oligomannosides for use in the methods ~~of the present invention~~ include, without limitation, an oligomannoside having the mannotetraose Man(1-3) Man(1-2) Man(1-2) Man. Such an oligomannoside can be purified from PPM as described in, *e.g.,* Faille *et al.*, *supra*. An exemplary neoglycolipid specific for ASCA can be constructed by releasing the oligomannoside from its respective PPM and subsequently coupling the released oligomannoside to 4-hexadecylaniline or the like.

3. Anti-Microbial Antibodies

[0166] The determination of anti-OmpC antibody levels in a sample is also useful in the present method ~~invention~~. As used herein, the term "anti-outer membrane protein C antibody" or "anti-OmpC antibody" includes antibodies directed to a bacterial outer membrane porin as described in, *e.g.,* PCT Patent Publication No. WO 01/89361. The term "outer membrane protein C" or "OmpC" refers to a bacterial porin that is immunoreactive with an anti-OmpC antibody.

[0167] The level of anti-OmpC antibody present in a sample from an individual can be determined using an OmpC protein or a fragment thereof such as an immunoreactive fragment thereof. Suitable OmpC antigens useful in determining anti-OmpC antibody levels in a sample include, without limitation, an OmpC protein, an OmpC polypeptide having substantially the same amino acid sequence as the OmpC protein, or a fragment thereof such as an immunoreactive fragment thereof. As used herein, an OmpC polypeptide generally describes polypeptides having an amino acid sequence with greater than about 50% identity, preferably greater than about 60% identity, more preferably greater than about 70% identity, still more preferably greater than about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% amino acid sequence identity with an OmpC protein, with the amino acid identity determined using a sequence alignment program such as CLUSTALW. Such antigens can be prepared, for example, by purification from enteric bacteria such as *E. coli*, by recombinant expression of a nucleic acid such as Genbank Accession No. K00541, by synthetic means such as solution or solid phase peptide synthesis, or by using phage display.

[0168] The determination of anti-I2 antibody levels in a sample is also useful in the present method ~~invention~~. As used herein, the term "anti-I2 antibody" includes antibodies directed to a microbial antigen sharing homology to bacterial transcriptional regulators as described in, *e.g.,* U.S. Patent No. 6,309,643. The term "I2" refers to a microbial antigen that is

immunoreactive with an anti-I2 antibody. The microbial I2 protein is a polypeptide of 100 amino acids sharing some similarity weak homology with the predicted protein 4 from *C. pasteurianum*, Rv3557c from *Mycobacterium tuberculosis*, and a transcriptional regulator from *Aquifex aeolicus*. The nucleic acid and protein sequences for the I2 protein are described in, *e.g.,* U.S. Patent No. 6,309,643.

[0169] The level of anti-I2 antibody present in a sample from an individual can be determined using an I2 protein or a fragment thereof such as an immunoreactive fragment thereof. Suitable I2 antigens useful in determining anti-I2 antibody levels in a sample include, without limitation, an I2 protein, an I2 polypeptide having substantially the same amino acid sequence as the I2 protein, or a fragment thereof such as an immunoreactive fragment thereof. Such I2 polypeptides exhibit greater sequence similarity to the I2 protein than to the *C. pasteurianum* protein 4 and include isotype variants and homologs thereof. As used herein, an I2 polypeptide generally describes polypeptides having an amino acid sequence with greater than about 50% identity, preferably greater than about 60% identity, more preferably greater than about 70% identity, still more preferably greater than about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% amino acid sequence identity with a naturally-occurring I2 protein, with the amino acid identity determined using a sequence alignment program such as CLUSTALW. Such I2 antigens can be prepared, for example, by purification from microbes, by recombinant expression of a nucleic acid encoding an I2 antigen, by synthetic means such as solution or solid phase peptide synthesis, or by using phage display.

[0170] The determination of anti-flagellin antibody levels in a sample is also useful in the present method ~~invention~~. As used herein, the term "anti-flagellin antibody" includes antibodies directed to a protein component of bacterial flagella as described in, *e.g.,* PCT Patent Publication No. WO 03/053220 and U.S. Patent Publication No. 20040043931. The term "flagellin" refers to a bacterial flagellum protein that is immunoreactive with an anti-flagellin antibody. Microbial flagellins are proteins found in bacterial flagellum that arrange themselves in a hollow cylinder to form the filament.

[0171] The level of anti-flagellin antibody present in a sample from an individual can be determined using a flagellin protein or a fragment thereof such as an immunoreactive fragment thereof. Suitable flagellin antigens useful in determining anti-flagellin antibody levels in a sample include, without limitation, a flagellin protein such as Cbir-1 flagellin,

flagellin X, flagellin A, flagellin B, fragments thereof, and combinations thereof, a flagellin polypeptide having substantially the same amino acid sequence as the flagellin protein, or a

fragment thereof such as an immunoreactive fragment thereof. As used herein, a flagellin polypeptide generally describes polypeptides having an amino acid sequence with greater than about 50% identity, preferably greater than about 60% identity, more preferably greater than about 70% identity, still more preferably greater than about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% amino acid sequence identity with a naturally-occurring flagellin protein, with the amino acid identity determined using a sequence alignment program such as CLUSTALW. Such flagellin antigens can be prepared, *e.g.,* by purification from bacterium such as *Helicobacter Bilis*, *Helicobacter mustelae*, *Helicobacter pylori*, *Butyrivibrio fibrisolvens*, and bacterium found in the cecum, by recombinant expression of a nucleic acid encoding a flagellin antigen, by synthetic means such as solution or solid phase peptide synthesis, or by using phage display.

D. Oxidative Stress Markers

[0172] The determination of markers of oxidative stress in a sample is also useful in the present method ~~invention~~. Non-limiting examples of markers of oxidative stress include those that are protein-based or DNA-based, which can be detected by measuring protein oxidation and DNA fragmentation, respectively. Other examples of markers of oxidative stress include organic compounds such as malondialdehyde.

[0173] Oxidative stress represents an imbalance between the production and manifestation of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Disturbances in the normal redox state of tissues can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA. Some reactive oxidative species can even act as messengers through a phenomenon called redox signaling.

[0174] ~~In certain embodiments, d~~Derivatives of reactive oxidative metabolites (DROMs), ratios of oxidized to reduced glutathione (Eh GSH), and/or ratios of oxidized to reduced cysteine (Eh CySH) can be used to quantify oxidative stress. *See, e.g.,* Neuman *et al.*, *Clin. Chem.*, 53:1652-1657 (2007). Oxidative modifications of highly reactive cysteine residues in proteins such as tyrosine phosphatases and thioredoxin-related proteins can also be detected or measured using a technique such as, *e.g.,* mass spectrometry (MS). *See, e.g.,* Naito *et al.*, *Anti-Aging Medicine*, 7 (5):36-44 (2010). Other markers of oxidative stress include protein-bound acrolein as described, *e.g.,* in Uchida *et al.*, *PNAS*, 95 (9) 4882-4887 (1998), the free oxygen radical test (FORT), which reflects levels of organic hydroperoxides, and the redox

potential of the reduced glutathione/glutathione disulfide couple, (Eh) GSH/GSSG. *See, e.g.,* Abramson *et al.*, *Atherosclerosis*, 178(1):115-21 (2005).

E. Cell Surface Receptors

[0175] The determination of cell surface receptors in a sample is also useful in the present methodinvention. The half-life of anti-TNFα drugs such as Remicade and Humira is significantly decreased in patients with a high level of inflammation. CD64, the high-affinity receptor for immunoglobulin (Ig) G1 and IgG3, is predominantly expressed by mononuclear phagocytes. Resting polymorphonuclear (PMN) cells scarcely express CD64, but the expression of this marker is upregulated by interferon and granulocyte-colony-stimulating factor acting on myeloid precursors in the bone marrow. Crosslinking of CD64 with IgG complexes exerts a number of cellular responses, including the internalization of immune complexes by endocytosis, phagocytosis of opsonized particles, degranulation, activation of the oxidative burst, and the release of cytokines.

[0176] As such, in certain aspects, the methods described herein utilize the application of an algorithm (*e.g.,* statistical analysis) to the presence, concentration level, and/or genotype determined for one or more cell surface receptors such as CD64 (*e.g.,* alone or in combination with biomarkers from other categories) to aid or assist in predicting disease course, selecting an appropriate anti-TNFα drug therapy, optimizing anti-TNFα drug therapy, reducing toxicity associated with anti-TNFα drug therapy, or monitoring the efficacy of therapeutic treatment with an anti-TNFα drug.

F. Signaling Pathways

[0177] The determination of signaling pathways in a sample is also useful in the present methodinvention. Polymorphonuclear (PMN) cell activation, followed by infltration into the intestinal mucosa (synovium for RA) and migration across the crypt epithelium is regarded as a key feature of IBD. It has been estimated by fecal indium-111-labeled leukocyte excretion that migration of PMN cells from the circulation to the diseased section of the intestine is increased by 10-fold or more in IBD patients. Thus, in certain aspects, measuring activation of PMN cells from blood or tissue inflammation by measuring signaling pathways using an assay such as the Collaborative Enzyme Enhanced Reactive ImmunoAssay (CEER) is an ideal way to understand inflammatory disease. The CEER technology is described in the following patent documents, which are each herein incorporated by reference in their entirety for all purposes: PCT Publication Nos. WO 2008/036802, WO 2009/012140, WO

2009/108637, WO 2010/132723, WO 2011/008990, and WO 2011/050069; and PCT Application No. PCT/US2011/066624.

[0178] As such, in certain aspects, the methods described herein utilize the application of an algorithm (*e.g.,* statistical analysis) to the presence, concentration level, and/or genotype determined for one or more signal transduction molecules in one or more signaling pathways (*e.g.,* alone or in combination with biomarkers from other categories) to aid or assist in predicting disease course, selecting an appropriate anti-TNFα drug therapy, optimizing anti-TNFα drug therapy, reducing toxicity associated with anti-TNFα drug therapy, or monitoring the efficacy of therapeutic treatment with an anti-TNFα drug. ~~In preferred embodiments,~~ ~~t~~The total level and/or activation (*e.g.,* phosphorylation) level of one or more signal transduction molecules in one or more signaling pathways ~~is~~ can be measured.

[0179] The term "signal transduction molecule" or "signal transducer" includes proteins and other molecules that carry out the process by which a cell converts an extracellular signal or stimulus into a response, typically involving ordered sequences of biochemical reactions inside the cell. Examples of signal transduction molecules include, but are not limited to, receptor tyrosine kinases such as EGFR (*e.g.,* EGFR/HER1/ErbB1, HER2/Neu/ErbB2, HER3/ErbB3, HER4/ErbB4), VEGFR1/FLT1, VEGFR2/FLK1/KDR, VEGFR3/FLT4, FLT3/FLK2, PDGFR (*e.g.,* PDGFRA, PDGFRB), c-KIT/SCFR, INSR (insulin receptor), IGF-IR, IGF-IIR, IRR (insulin receptor-related receptor), CSF-1R, FGFR 1-4, HGFR 1-2, CCK4, TRK A-C, c-MET, RON, EPHA 1-8, EPHB 1-6, AXL, MER, TYRO3, TIE 1-2, TEK, RYK, DDR 1-2, RET, c-ROS, V-cadherin, LTK (leukocyte tyrosine kinase), ALK (anaplastic lymphoma kinase), ROR 1-2, MUSK, AATYK 1-3, and RTK 106; truncated forms of receptor tyrosine kinases such as truncated HER2 receptors with missing amino-terminal extracellular domains (*e.g.,* p95ErbB2 (p95m), p110, p95c, p95n, *etc.*), truncated cMET receptors with missing amino-terminal extracellular domains, and truncated HER3 receptors with missing amino-terminal extracellular domains; receptor tyrosine kinase dimers (*e.g.,* p95HER2/HER3; p95HER2/HER2; truncated HER3 receptor with HER1, HER2, HER3, or HER4; HER2/HER2; HER3/HER3; HER2/HER3; HER1/HER2; HER1/HER3; HER2/HER4; HER3/HER4; *etc.*); non-receptor tyrosine kinases such as BCR-ABL, Src, Frk, Btk, Csk, Abl, Zap70, Fes/Fps, Fak, Jak, Ack, and LIMK; tyrosine kinase signaling cascade components such as AKT (*e.g.,* AKT1, AKT2, AKT3), MEK (MAP2K1), ERK2 (MAPK1), ERK1 (MAPK3), PI3K (*e.g.,* PIK3CA (p110), PIK3R1 (p85)), PDK1, PDK2, phosphatase and tensin homolog (PTEN), SGK3, 4E-BP1, P70S6K (*e.g.,* p70 S6 kinase splice variant alpha I), protein tyrosine phosphatases (*e. g.,* PTP1B, PTPN13, BDP1, *etc.*), RAF, PLA2,

GRB2 (p-GRB2), CRKL (p-CRKL), PLCγ (p-PLCγ), PKC (*e.g.,* p-PKCα, p-PKCβ, p-PKCδ), adducin (p-adducin), RB1 (p-RB1), and PYK2 (p-PYK2).

[0181] The following tables provide additional examples of signal transduction molecules for which total levels and/or activation (*e.g.,* phosphorylation) levels can be determined in a sample (*e.g.,* alone or in combination with biomarkers from other categories) to aid or assist in predicting disease course, selecting an appropriate anti-TNFα drug therapy, optimizing anti-TNFα drug therapy, reducing toxicity associated with anti-TNFα drug therapy, or monitoring the efficacy of therapeutic treatment with an anti-TNFα drug.

Table 1

Total / Phospho Assays		Phospho sites
VEGFR2 Total	VEGFR2 Phospho	Y951, 1212
Erk Total	Erk Phospho	T202/Y204
Akt Total	Akt Phospho	T308, S473
MEK Total	MEK Phospho	S217/221
MEK Total	MEK Phospho	S217/221
P70S6K Total	P70S6K Phospho	T389 (T229)
PTEN Total		
VEGFR1 (T)	VEGFR1 Phospho	
SGK total	SGK phospho	T320, S486
CRKL Total	CRKL Phospho	Y207
SRC Total	SRC Phospho	Y 416, 527
FAK Total	FAK Phospho	Y397
BCR Total	BCR Phospho	
PI3K activated	PI3K complexed	P85 Y688
4EBP1	4EBP1 phospho	T70, T37, T46
PRAS40	PRAS40 phospho	T246

Table 2

Total / Phospho Assays		Phospho sites
TIE Total	TIE- 2 Phospho	Y992 (S1119)
Jak 2 Total	JAK 2 Phospho	Y1007/1008
STAT5 Total	STAT 5 Phospho	Y694/699
STAT 3Total	STAT 3 Phospho	Y705
FGFR1 total	FGFR1 Phospho	Y 653, 766
FGFR2 total	FGFR 2 Phospho	Y653
FGFR3 total	FGFR 3 Phospho	
FGFR4 total	FGFR 4 Phospho	
Axl total	Axl Phospho	Y702
BAD total	BAD Phospho	(S112) (S136)
RSK total	RSK Phospho	(T359/S363)
PDK total	PDK 1 Phospho	(S241)
JAK 1 and 3 total	JAK 1 and 3 Phospho	
TSC2 total	TSC 2 Phospho	S664, S939
S6RP Total	S6RP phospho	S235/236

G. Genetic Markers

[0182] The determination of the presence or absence of allelic variants (*e.g.,* SNPs) in one or more genetic markers in a sample (*e.g.,* alone or in combination with biomarkers from other categories) is also useful in the methods ~~of the present invention~~ to aid or assist in predicting disease course, selecting an appropriate anti-TNFα drug therapy, optimizing anti-TNFα drug therapy, reducing toxicity associated with anti-TNFα drug therapy, or monitoring the efficacy of therapeutic treatment with an anti-TNFα drug.

[0183] Non-limiting examples of genetic markers include, but are not limited to, any of the inflammatory pathway genes and corresponding SNPs that can be genotyped as set forth in

TNFRSF6B	rs2160322
TNFRSF6B	rs2315008
TNFRSF6B	rs4809330
PSMG1	rs2094871
PSMG1	rs2836878
PTPN2	rs2542151
MST1/3p21	rs9858542
MST1/3p21	rs3197999
SLC22A23	rs17309827
MHC	rs660895
XBP1	rs35873774
ICOSLG1	rs762421
BTLN2	rs3763313
BTLN2	rs2395185
BTLN2	rs9268480
ATG5	rs7746082
CUL2,CREM	rs17582416
CARD9	rs4077515
ORMDL3	rs2872507
ORMDL3	rs2305480

[0184] Additional SNPs useful in the present method ~~invention~~ include, *e.g.,* rs2188962, rs9286879, rs11584383, rs7746082, rs1456893, rs1551398, rs17582416, rs3764147, rs1736135, rs4807569, rs7758080, and rs8098673. *See, e.g.,* Barrett *et al.*, *Nat. Genet.*, 40:955-62 (2008).

1. NOD2/CARD15

[0185] The determination of the presence or absence of allelic variants such as SNPs in the NOD2/CARD15 gene is particularly useful in the present method ~~invention~~. As used herein, the term "NOD2/CARD15 variant" or "NOD2 variant" includes a nucleotide sequence of a NOD2 gene containing one or more changes as compared to the wild-type NOD2 gene or an amino acid sequence of a NOD2 polypeptide containing one or more changes as compared to the wild-type NOD2 polypeptide sequence. NOD2, also known as CARD15, has been localized to the IBD1 locus on chromosome 16 and identified by positional-cloning (Hugot *et al.*, *Nature*, 411:599-603 (2001)) as well as a positional candidate gene strategy (Ogura *et al.*, *Nature*, 411:603-606 (2001); Hampe *et al.*, *Lancet*, 357:1925-1928 (2001)). The IBD1 locus has a high multipoint linkage score (MLS) for inflammatory bowel disease (MLS=5.7 at marker D16S411 in 16q12). *See, e.g.,* Cho *et al.*, *Inflamm. Bowel Dis.*, 3:186-190 (1997);

Akolkar *et al.*, *Am. J. Gastroenterol.*, 96:1127-1132 (2001); Ohmen *et al.*, *Hum. Mol. Genet.*, 5:1679-1683 (1996); Parkes *et al.*, *Lancet*, 348:1588 (1996); Cavanaugh *et al.*, *Ann. Hum. Genet.*, 62:291-8 (1998); Brant *et al.*, *Gastroenterology*, 115:1056-1061 (1998); Curran *et al.*, *Gastroenterology*, 115:1066-1071 (1998); Hampe *et al.*, *Am. J. Hum. Genet.*, 64:808-816 (1999); and Annese *et al.*, *Eur. J. Hum. Genet.*, 7:567-573 (1999).

[0186] The mRNA (coding) and polypeptide sequences of human NOD2 are set forth in, *e.g.*, Genbank Accession Nos. NM_022162 and NP_071445, respectively. In addition, the complete sequence of human chromosome 16 clone RP11-327F22, which includes NOD2, is set forth in, *e.g.*, Genbank Accession No. AC007728. Furthermore, the sequence of NOD2 from other species can be found in the GenBank database.

[0187] The NOD2 protein contains amino-terminal caspase recruitment domains (CARDs), which can activate NF-kappa B (NF-kB), and several carboxy-terminal leucine-rich repeat domains (Ogura *et al.*, *J. Biol. Chem.*, 276:4812-4818 (2001)). NOD2 has structural homology with the apoptosis regulator Apaf-1/CED-4 and a class of plant disease resistant gene products (Ogura *et al.*, *supra*). Similar to plant disease resistant gene products, NOD2 has an amino-terminal effector domain, a nucleotide-binding domain and leucine rich repeats (LRRs). Wild-type NOD2 activates nuclear factor NF-kappa B, making it responsive to bacterial lipopolysaccharides (LPS; Ogura *et al.*, *supra*; Inohara *et al.*, *J. Biol. Chem.*, 276:2551-2554 (2001). NOD2 can function as an intercellular receptor for LPS, with the leucine rich repeats required for responsiveness.

[0188] Variations at three single nucleotide polymorphisms in the coding region of NOD2 have been previously described. These three SNPs, designated R702W ("SNP 8"), G908R ("SNP 12"), and 1007fs ("SNP 13"), are located in the carboxy-terminal region of the NOD2 gene (Hugot *et al.*, *supra*). A further description of SNP 8, SNP 12, and SNP 13, as well as additional SNPs in the NOD2 gene suitable for use in the method ~~invention~~, can be found in, *e.g.*, U.S. Patent Nos. 6,835,815; 6,858,391; and 7,592,437; and U.S. Patent Publication Nos. 20030190639, 20050054021, and 20070072180.

[0189] ~~In some embodiments, a~~A NOD2 variant ~~is~~ can be located in a coding region of the NOD2 locus, for example, within a region encoding several leucine-rich repeats in the carboxy-terminal portion of the NOD2 polypeptide. Such NOD2 variants located in the leucine-rich repeat region of NOD2 include, without limitation, R702W ("SNP 8") and G908R ("SNP 12"). A NOD2 variant useful in the method ~~invention~~ can also encode a

NOD2 polypeptide with reduced ability to activate NF-kappa B as compared to NF-kappa B activation by a wild-type

NOD2 polypeptide. As a non-limiting example, the NOD2 variant 1007fs ("SNP 13") results in a truncated NOD2 polypeptide which has reduced ability to induce NF-kappa B in response to LPS stimulation (Ogura *et al.*, *Nature*, 411:603-606 (2001)).

[0190] A NOD2 variant useful in the method ~~invention~~ can be, for example, R702W, G908R, or 1007fs. R702W, G908R, and 1007fs are located within the coding region of NOD2. ~~In one embodiment, a~~A method of the ~~invention is~~ can be practiced with the R702W NOD2 variant. As used herein, the term "R702W" includes a single nucleotide polymorphism within exon 4 of the NOD2 gene, which occurs within a triplet encoding amino acid 702 of the NOD2 protein. The wild-type NOD2 allele contains a cytosine (c) residue at position 138,991 of the AC007728 sequence, which occurs within a triplet encoding an arginine at amino acid702. The R702W NOD2 variant contains a thymine (t) residue at position 138,991 of the AC007728 sequence, resulting in an arginine (R) to tryptophan (W) substitution at amino acid 702 of the NOD2 protein. Accordingly, this NOD2 variant is denoted "R702W" or "702W" and can also be denoted "R675W" based on the earlier numbering system of Hugot *et al.*, *supra*. In addition, the R702W variant is also known as the "SNP 8" allele or a "2" allele at SNP 8. The NCBI SNP ID number for R702W or SNP 8 is rs2066844. The presence of the R702W NOD2 variant and other NOD2 variants can be conveniently detected, for example, by allelic discrimination assays or sequence analysis.

[0191] A method ~~of the invention~~ can also be practiced with the G908R NOD2 variant. As used herein, the term "G908R" includes a single nucleotide polymorphism within exon 8 of the NOD2 gene, which occurs within a triplet encoding amino acid 908 of the NOD2 protein. Amino acid 908 is located within the leucine rich repeat region of the NOD2 gene. The wild-type NOD2 allele contains a guanine (g) residue at position 128,377 of the AC007728 sequence, which occurs within a triplet encoding glycine at amino acid 908. The G908R NOD2 variant contains a cytosine (c) residue at position 128,377 of the AC007728 sequence, resulting in a glycine (G) to arginine (R) substitution at amino acid 908 of the NOD2 protein. Accordingly, this NOD2 variant is denoted "G908R" or "908R" and can also be denoted "G881R" based on the earlier numbering system of Hugot *et al.*, *supra*. In addition, the G908R variant is also known as the "SNP 12" allele or a "2" allele at SNP 12. The NCBI SNP ID number for G908R SNP 12 is rs2066845.

[0192] A method ~~of the invention~~ can also be practiced with the 1007fs NOD2 variant. This variant is an insertion of a single nucleotide that results in a frame shift in the tenth

leucine-rich repeat of the NOD2 protein and is followed by a premature stop codon. The resulting truncation of the NOD2 protein appears to prevent activation of NF-kappaB in

limiting examples, a JW1 variant allele can have a cytosine (c) to adenine (a), cytosine (c) to guanine (g), or cytosine (c) to thymine (t) substitution at nucleotide 158 of intron 8. ~~In one~~

~~embodiment, t~~The JW1 variant allele ~~is~~ can be a change from a cytosine (c) to a thymine (t) at nucleotide 158 of NOD2 intron 8.

[0196] The term "JW15 variant allele" includes a genetic variation in the 3' untranslated region of NOD2 at nucleotide position 118,790 of the AC007728 sequence. The genetic variation at nucleotide 118,790 can be, but is not limited to, a single nucleotide substitution, multiple nucleotide substitutions, or a deletion or insertion of one or more nucleotides. The wild-type sequence has an adenine (a) at position 118,790. As non-limiting examples, a JW15 variant allele can have an adenine (a) to cytosine (c), adenine (a) to guanine (g), or adenine (a) to thymine (t) substitution at nucleotide 118,790. ~~In one embodiment, t~~The JW15 variant allele ~~is~~ can be a change from an adenine (a) to a cytosine (c) at nucleotide 118,790.

[0197] As used herein, the term "JW16 variant allele" includes a genetic variation in the 3' untranslated region of NOD2 at nucleotide position 118,031 of the AC007728 sequence. The genetic variation at nucleotide 118,031 can be, but is not limited to, a single nucleotide substitution, multiple nucleotide substitutions, or a deletion or insertion of one or more nucleotides. The wild-type sequence has a guanine (g) at position 118,031. As non-limiting examples, a JW16 variant allele can have a guanine (g) to cytosine (c), guanine (g) to adenine (a), or guanine (g) to thymine (t) substitution at nucleotide 118,031. ~~In one embodiment, t~~The JW16 variant allele ~~is~~ can be a change from a guanine (g) to an adenine (a) at nucleotide 118,031.

[0198] The term "JW17 variant allele" includes a genetic variation in the 5' untranslated region of NOD2 at nucleotide position 154,688 of the AC007728 sequence. The genetic variation at nucleotide 154,688 can be, but is not limited to, a single nucleotide substitution, multiple nucleotide substitutions, or a deletion or insertion of one or more nucleotides. The wild-type sequence has a cytosine (c) at position 154,688. As non-limiting examples, a JW17 variant allele can have a cytosine (c) to guanine (g), cytosine (c) to adenine (a), or cytosine (c) to thymine (t) substitution at nucleotide 154,688. ~~In one embodiment, t~~The JW17 variant allele ~~is~~ can be a change from a cytosine (c) to a thymine (t) at nucleotide 154,688.

[0199] As used herein, the term "JW18 variant allele" includes a genetic variation in the 5' untranslated region of NOD2 at nucleotide position 154,471 of the AC007728 sequence. The genetic variation at nucleotide 154,471 can be, but is not limited to, a single nucleotide substitution, multiple nucleotide substitutions, or a deletion or insertion of one or more nucleotides. The wild-type sequence has a cytosine (c) at position 154,471. As non-limiting examples, a JW18 variant allele can have a cytosine (c) to guanine (g), cytosine (c) to adenine

(a), or cytosine (c) to thymine (t) substitution at nucleotide 154,471. ~~In one embodiment,~~ ~~t~~The JW18 variant allele ~~is~~ can be a change from a cytosine (c) to a thymine (t) at nucleotide 154,471.

[0200] It is understood that the methods ~~of the invention~~ can be practiced with these or other NOD2 variant alleles located in a coding region or non-coding region (*e.g.,* intron or promoter region) of the NOD2 locus. It is further understood that the methods ~~of the invention~~ can involve determining the presence of one, two, three, four, or more NOD2 variants, including, but not limited to, the SNP 8, SNP 12, and SNP 13 alleles, and other coding as well as non-coding region variants.

V. Examples

[0201] The present method ~~invention~~ will be described in greater detail by way of specific examples. The following examples are offered for illustrative purposes~~, and are not intended to limit the invention in any manner~~. Those of skill in the art will readily recognize a variety of noncritical parameters which can be changed or modified to yield essentially the same results.

Example 1. Novel Mobility Shift Assay for Measuring Levels of anti-TNFα Biologics.

[0202] This example illustrates a novel homogeneous assay for measuring anti-TNFα drug concentration in a patient sample (*e.g.,* serum) using size exclusion chromatography to detect the binding of the anti-TNFα drug to fluorescently labeled TNFα. The assay is advantageous because it obviates the need for wash steps, uses fluorophores that allow for detection on the visible and/or IR spectra which decreases background and serum interference issues, increases the ability to detect anti-TNFα drugs in patients with a low titer due to the high sensitivity of fluorescent label detection, and occurs as a liquid phase reaction, thereby reducing the chance of any changes in the epitope by attachment to a solid surface such as an ELISA plate.

[0203] In one exemplary method ~~embodiment~~, TNFα is labeled with a fluorophore (*e.g.,* $Alexa_{647}$), wherein the fluorophore can be detected on either or both the visible and IR spectra. The labeled TNFα is incubated with human serum in a liquid phase reaction to allow the anti-TNFα drug present in the serum to bind. The labeled TNFα can also be incubated with known amounts of the anti-TNFα drug in a liquid phase reaction to create a standard curve. Following incubation, the samples are loaded directly onto a size exclusion column. Binding of the anti-TNFα drug to the labeled TNFα results in a leftward shift of the peak

compared to labeled TNFα alone. The concentration of the anti-TNFα drug present in the serum sample can then be compared to the standard curve and controls.

[0204] Figure 1 shows an example of the assay of the present method~~invention~~ wherein size exclusion HPLC is used to detect the binding between TNFα-Alexa$_{647}$ and HUMIRA™ (adalimumab). As shown in Figure 1, the binding of HUMIRA™ to TNFα-Alexa$_{647}$ caused a shift of the TNFα-Alexa$_{647}$ peak to the left.

[0205] Figure 2 shows dose response curves of HUMIRA™ binding to TNFα-Alexa$_{647}$. In particular, Figure 2A shows that HUMIRA™ dose-dependently increased the shift of TNFα-Alexa$_{647}$ in the size exclusion chromatography assay. Figure 2B shows that the presence of 1% human serum did not have a significant effect on the shift of TNFα-Alexa$_{647}$ in the size exclusion chromatography assay. Figure 2C shows that the presence of pooled RF-positive serum did not have a significant effect on the shift of TNFα-Alexa$_{647}$ in the size exclusion chromatography assay.

[0206] As such, this example demonstrates the utility of the present method ~~invention~~ in monitoring patients receiving an anti-TNFα drug such as HUMIRA™: (1) to guide in the determination of the appropriate drug dosage; (2) to evaluate drug pharmacokinetics, *e.g.,* to determine whether the drug is being cleared from the body too quickly; and (3) to guide treatment decisions, *e.g.,* whether to switch from the current anti-TNFα drug to a different TNFα inhibitor or to another type of therapy.

Example 2. Novel Mobility Shift Assay for Measuring HACA and HAHA Levels.

[0207] This example illustrates a novel homogeneous assay for measuring autoantibody (*e.g.,* HACA and/or HAHA) concentrations in a patient sample (*e.g.,* serum) using size exclusion chromatography to detect the binding of these autoantibodies to fluorescently labeled anti-TNFα drug. The assay is advantageous because it obviates the need for wash steps which remove low affinity HACA and HAHA, uses fluorophores that allow for detection on the visible and/or IR spectra which decreases background and serum interference issues, increases the ability to detect HACA and HAHA in patients with a low titer due to the high sensitivity of fluorescent label detection, and occurs as a liquid phase reaction, thereby reducing the chance of any changes in the epitope by attachment to a solid surface such as an ELISA plate.

[0208] The clinical utility of measuring autoantibodies (*e.g.,* HACA, HAHA, *etc.*) that are generated against TNFα inhibitors is illustrated by the fact that HACAs were detected in 53%, 21%, and 7% of rheumatoid arthritis patients treated with 1 mg/kg, 3 mg/kg, and 10 mg/kg infliximab. When infliximab was combined with methotrexate, the incidence of

antibodies was lower 15%, 7%, and 0%, which indicates that concurrent immunosuppressive therapy is effective in lowering anti-drug responses, but also indicates that a high dose of anti-TNFα antibody might lead to tolerance. In Crohn's disease, a much higher incidence was reported; after the fifth infusion, 61% of patients had HACA. The clinical response was shortened when HACAs were present. *See,* Rutgeerts, *N. Engl. J. Med.*, 348:601-608 (2003). A retrospective study of infliximab and HACA levels measured over a 3 year period from 2005 to 2008 in 155 patients demonstrated that HACAs were detected in 22.6% (N = 35) of patients with inflammatory bowel disease. *See,* Afif *et al.*, "Clinical Utility of Measuring Infliximab and Human Anti-Chimeric Antibody Levels in Patients with Inflammatory Bowel Disease"; paper presented at Digestive Disease Week; May 30-June 3, 2009; Chicago, IL. The authors concluded that changing treatment based on clinical symptoms alone may lead to inappropriate management.

[0209] The homogeneous mobility shift assay is advantageous over current methods such as the bridging assay shown in Figure 3 for measuring autoantibody (*e.g.,* HACA and/or HAHA) concentrations in a patient sample because the inventive method is capable of measuring the concentration of autoantibodies such as HACA without non-specific binding and solid phase interference from the ELISA plate, without interference from the anti-TNFα drug (*e.g.,* with the bridging assay, HACA measurements must be taken at anti-TNFα drug trough levels), and without any dependency on the multivalency of the antibody (*e.g.,* IgG4 antibodies are not detected using the bridging assay because IgG4 antibodies are bispecific and cannot cross-link the same antigen). As such, the present method ~~invention~~ has at least the following advantages over current methods: avoids attachment of antigens to solid surfaces (denaturation avoided); eliminates the IgG4 effect; overcomes therapeutic antibody trough issues; detects antibodies with weak affinities; eliminates non-specific binding of irrelevant IgGs; and increases the sensitivity and specificity of detection.

[0210] In one exemplary method ~~embodiment~~, an anti-TNFα drug (*e.g.,* REMICADE™) is labeled with a fluorophore (*e.g.,* $Alexa_{647}$), wherein the fluorophore can be detected on either or both the visible and IR spectra. The labeled anti-TNFα drug is incubated with human serum in a liquid phase reaction to allow HACA and HAHA present in the serum to bind. The labeled anti-TNFα drug can also be incubated with known amounts of an anti-IgG antibody in a liquid phase reaction to create a standard curve. Following incubation, the samples are loaded directly onto a size exclusion column. Binding of the autoantibodies to the labeled anti-TNFα drug results in a leftward shift of the peak compared to labeled drug alone. The concentration of HACA and HAHA present in the serum sample can then be compared to the

standard curve and controls. Figure 4 illustrates an exemplary outline of the autoantibody detection assays ~~of the present invention~~ for measuring the concentrations of HACA/HAHA generated against REMICADE™. In certain instances, high HACA/HAHA levels indicate that the current therapy with REMICADE™ should be switched to another anti-TNFα drug such as HUMIRA™.

[0211] The principle of this assay is based on the mobility shift of the antibody bound $Alexa_{647}$-labeled Remicade complex versus free $Alexa_{647}$-labeled Remicade on size exclusion- high performance liquid chromatography (SE-HPLC) due to the increase in molecular weight of the complex.

[0212] The chromatography in this example was performed on an Agilent-1200 HPLC System, using a Bio-Sep 300x7.8 mm SEC-3000 column (Phenomenex) with a molecular weight fractionating range of 5,000 – 700,000 and a mobile phase of 1X PBS, pH 7.4, at a flow-rate of 0.5 mL/min with UV detection at 650 nm. A 100 µL sample volume is loaded onto the column for each analysis.

[0213] The antibody bound $Alexa_{647}$-labeled Remicade complex is formed by incubating a known amount of the antibody and $Alexa_{647}$-labeled Remicade in the 1X PBS, pH 7.3, elution buffer at room temperature for 1 hour before SE-HPLC analysis.

[0214] Figure 5 shows a dose response analysis of anti-human IgG antibody binding to REMICADE™-$Alexa_{647}$ as detected using the size exclusion chromatography assay ~~of the present invention~~. The binding of anti-IgG antibody to REMICADE™-$Alexa_{647}$ caused a shift of the REMICADE™-$Alexa_{647}$ peak to the left. Figure 6 shows a second dose response analysis of anti-human IgG antibody binding to REMICADE™-$Alexa_{647}$ as detected using the size exclusion chromatography assay ~~of the present invention~~. Higher amounts of anti-IgG antibody resulted in a dose-dependent increase in the formation of anti-IgG/REMICADE™-$Alexa_{647}$ complexes, as indicated by a shift of the REMICADE™-$Alexa_{647}$ peak to the left. Figure 7 shows dose response curves of anti-IgG antibody binding to REMICADE™-$Alexa_{647}$.

[0215] Figure 8 shows REMICADE™-$Alexa_{647}$ immunocomplex formation in normal human serum and HACA positive serum as detected using the size exclusion chromatography assay ~~of the present invention~~ with 100 µl of injected sample. As shown in Figure 8, the binding of HACA present in patient samples to REMICADE™-$Alexa_{647}$ caused a shift of the REMICADE™-$Alexa_{647}$ peak to the left. As such, the size exclusion chromatography assay

~~of the invention~~ is particularly advantageous because it measures HACA in the presence of REMICADE™, can be utilized while the patient is on therapy, measures both weak and strong HACA binding, is a mix and read mobility shift assay, and can be extended to other approaches which use labeled REMICADE™ to equilibrate with HACA and REMICADE™.

[0216] Figure 9 provides a summary of HACA measurements from 20 patient serum samples that were performed using the bridging assay or the mobility shift assay ~~of the present invention~~. This comparative study demonstrates that the present methods have increased sensitivity over current methods because 3 samples that were negative for HACA as measured using the bridging assay were actually HACA positive when measured using the mobility shift assay ~~of the present invention~~ (*see,* Patient # SK07070305, SK07070595, and SK07110035).

[0217] As such, this example demonstrates the utility of the present method ~~invention~~ in monitoring patients receiving an anti-TNFα drug (*e.g.,* REMICADE™) to detect the presence or level of autoantibodies (*e.g.,* HACA and/or HAHA) against the drug, because such immune responses can be associated with hypersensitive reactions and dramatic changes in pharmacokinetics and biodistribution of the anti-TNFα drug that preclude further treatment with the drug.

[0218] In conclusion, Examples 1 and 2 demonstrate that TNFα and anti-TNFα antibodies can be efficiently labeled with $Alexa_{647}$. When labeled TNFα-$Alexa_{647}$ was incubated with anti-TNFα antibodies, the retention time of the labeled TNFα/anti-TNFα antibody complex was shifted, and the amount of anti-TNFα antibody that caused the shift could be quantitated with HPLC. Furthermore, when labeled anti-TNFα antibody was incubated with anti-human IgG antibody, the retention time of the labeled anti-TNFα antibody/anti-IgG antibody complex was shifted, and the amount of anti-IgG antibody that caused the shift could be quantitated with HPLC. Moreover, low serum content in the assay system was shown to have little effect on HPLC analysis. Finally, a standard curve could be generated for the anti-TNFα antibody and HACA/HAHA assays and could be used to quantitate patient serum anti-TNFα antibody or HACA/HAHA levels. Advantageously, the present method ~~invention~~ provides in certain aspects a mobility shift assay, such as a homogeneous mix and read assay developed to measure both drug and antibodies against the drug. A standard curve was generated for the anti-TNFα biologic Remicade and Humira and also for the HACA antibodies against Remicade. The mobility shift assay format, unlike ELISA, eliminates coating of antigens to solid surface and is not affected by non-specific binding of irrelevant

IgGs. The assay format is simple, but very sensitive and can be used to detect all anti-TNFα biologic drugs (*e.g.,*

determined by the Bridge assay. HACA levels were found to be increased in 11 patients during the course of IFX treatment while the IFX levels were found to be significantly decreased .

[0222] Conclusions: A novel non-radiolabeled, liquid-phase, mobility shift assay has been developed to measure the IFX and HACA levels in serum from patients treated with IFX. The assay has high sensitivity and accuracy, and the obtained results were reproducible. This novel assay can advantageously be used to measure HACA and IFX levels while patients are on therapy.

INTRODUCTION

[0223] Tumor necrosis factor-alpha (TNFα) plays a pivotal role in the pathogenesis of autoimmune diseases such as Crohn's disease (CD) and rheumatoid arthritis (RA). It is well documented that blocking TNFα with therapeutic antibodies such as Infliximab (human-murine chimeric monoclonal IgG1κ) or adalimumab (fully human monoclonal antibody) reduces disease activity in CD and RA. However, about 30-40% of the patients do not respond to anti-TNFα therapy and some patients need higher doses or dosing frequency adjustments due to lack of sufficient response. Differences of drug bioavailability and pharmacokinetics in individual patients may contribute to the failure of the treatment. Immunogenicity of the drugs, which causes patients to develop HACA/HAHA, could result in a range of adverse reactions from mild allergic response to anaphylactic shock. These problems are now recognized by many investigators, drug-controlling agencies, health insurance companies, and drug manufacturers. Furthermore, many patients with secondary response failure to one anti-TNFα drug benefit from a switch to other anti-TNFα drugs, suggesting a role of neutralizing antibodies directed specifically against the protein used for treatment (Radstake *et al.*, *Ann. Rheum. Dis.*, 68(11):1739-45 (2009)). Monitoring of patients for drug and HACA/HAHA levels is therefore warranted so that drug administration can be tailored to the individual patient and prolonged therapies can be given effectively and economically with little or no risk to patients (Bendtzen *et al.*, *Scand. J. Gastroenterol.*, 44(7):774-81 (2009)).

[0224] Several enzyme-linked immunoassays have been used to assess the circulating levels of drugs and HACA/HAHA. Figure 10 provides a summary of the current assays available for the measurement of HACA in comparison to the novel HACA assay of the present method ~~invention~~. One of the limitations of current methodologies is that antibody levels are difficult to measure when there is a measurable amount of drug in the circulation. In contrast

to current solid-phase methods for detecting HACA in which measurements can only be performed at least 8 weeks following a dose of IFX, the novel assay of the present methodinvention is a non-radiolabeled, liquid-phase, size-exclusion (SE)-HPLC assay that is capable of measuring HACA and IFX levels in serum from patients while being treated with IFX.

[0225] The following are rationales for measuring the serum concentrations of anti-TNFα biologic drugs and antibodies against TNFα biologic drugs in patients: (1) for PK studies in clinical trials; (2) it may be required by the FDA during clinical trials to monitor a patient's immune response to the biologic drug; (3) to monitor a patient's response to the biologic drug by measuring HACA or HAHA to guide the drug dosage for each patient; and (4) for use as a guide for switching to a different biologic drug when the initial drug fails.

METHODS

[0226] *SE-HPLC analysis of Infliximab (IFX) levels in patient serum.* Human recombinant TNFα was labeled with a fluorophore ("Fl" such as, *e.g.,* Alexa Fluor® 488) according to the manufacture's instructions. Labeled TNFα was incubated with different amounts of IFX or patient serum for one hour at room temperature. Samples of 100 µL volume were analyzed by size-exclusion chromatography on an HPLC system. FLD was used to monitor the free TNFα-Fl and the bound TNFα-Fl immuno-complex based on their retention times. Serum IFX levels were calculated from the standard curve.

[0227] *SE-HPLC analysis of HACA levels in patient serum.* Purified IFX was labeled with Fl. Labeled IFX was incubated with different dilutions of pooled HACA-positive serum or diluted patient serum for one hour at room temperature. Samples of 100 µL volume were analyzed by size-exclusion chromatography on an HPLC system. FLD was used to monitor the free IFX-Fl and the bound IFX-Fl immuno-complex based on their retention times. The ratio of bound and free IFX- Fl was used to determine the HACA level.

[0228] *Mobility Shift Assay Procedure to Measure HACA in Serum.* The principle of this assay is based on the mobility shift of the HACA bound Fl-labeled Infliximab (IFX) complex versus free Fl-labeled IFX on size exclusion-high performance liquid chromatography (SE-HPLC) due to the increase in molecular weight of the complex. The chromatography is performed in an Agilent-1200 HPLC System, using a Bio-Sep 300x7.8 mm SEC-3000 column (Phenomenex) with a molecular weight fractionating range of 5,000-700,000 and a mobile phase of 1X PBS, pH 7.3, at a flow-rate of 0.5-1.0 mL/min with FLD detection. A 100 µL sample volume is loaded onto the column for each analysis. The HACA bound Fl-

labeled IFX complex is formed by incubating serum from IFX treated patient and Fl-labeled IFX in the 1X PBS, pH 7.4, elution buffer at room temperature for 1 hour before SE-HPLC analysis. The assay was also run in the presence of varying interference agents, such as rheumatoid factor and TNF-β, in order to validate the assay.

RESULTS

[0229] Figure 11 shows the separation of the HACA bound IFX-Fl complex from the free IFX-Fl due to the mobility shift of the high molecular weight complex. As seen in panels **c** and **d**, the retention time of the fluorescent peak shifted from 21.8 min to 15.5-19.0 min. The more the HACA is present in the reaction mixture, the less the free IFX-Fl remains in the chromatogram and the more the immuno-complex is formed. Figure 12 shows the dose-response curves of the fluorescent peak shift caused by the addition of HACA. Using the HACA positive sample, we could detect the peak shift with 1:1000 dilutions of the serum.

[0230] Figure 13 shows the separation of the IFX bound TNFα-Fl complex from the free TNFα-Fl due to the mobility shift of the high molecular weight complex. As seen in panels **c** and **d**, the retention time of the fluorescent peak shifted from 24 min to 13-19.5 min. The more the IFX is present in the reaction mixture, the less the free TNFα-Fl remains in the chromatogram and the more the immuno-complex is formed. Figure 14 shows the dose-response curves of the TNFα-Fl peak shift caused by the addition of IFX. Based on the added IFX, the detection limit is 10 ng/mL of IFX in serum.

[0231] The novel mobility shift assay ~~of the present invention~~ was validated by testing serum samples from HACA positive and negative patients measured by the Bridge assay (Table 4). Using this assay, we have analyzed serum samples from 50 healthy subjects and 117 IBD patients treated with IFX. All 50 healthy subject samples have an IFX level below the limit of detection, whereas 65 of the patient samples have an average IFX concentration of 11.0 µg/ml. Table 5 shows the HACA levels in the serum of healthy controls and IBD patients treated with IFX measured by the Bridge assay and the mobility shift assay. The Bridge assay detected less HACA-positive patients than the mobility shift assay and more false negatives as well as more false positives.

Table 4. Correlation of Relative HACA Levels in Patient Serum from Strong Positive and Negative on Bridge Assay to SE-HPLC Assay.

	Bridge assay	HPLC shift assay	Correlation
Positive	82	81	99%
Negative	12	12	100%

Table 5. Patient Sample Analysis on Serum Levels of HACA with Bridge Assay (Cut Off 1.69 µg/ml) and HPLC Shift Assay (Cut Off 0.19, Ratio of Bound and Free IFX).

	Subjects (n)	HACA Positive		Bridge Assay	
		Bridge Assay	HPLC Assay	False Negative	False Positive
Healthy Control	50	N/A	0	N/A	N/A
Patient treated with IFX	117	24 (20.5%)	33 (28.2%)	9 (High IFX)	9

False negative results are caused by patient serum containing high levels of IFX which interferes with the Bridge assay on HACA determination while the SE-HPLC assay is not affected. False positive results are caused by patient serum containing high levels of non-specific interference substance which may interfere with the Bridge assay.

[0232] Figure 15 shows the relationship of the HACA level and IFX concentration in IBD patients during the course of IFX treatment. HACA could be detected as early as V10 (30 Weeks) and continued to increase in some patients during IFX treatment. Figure 16 shows that HACA can be detected in the presence of IFX using the assay ~~of the present invention~~. A higher level of HACA in the serum was associated with a lower level of IFX that could be detected (*e.g.,* reduced the bioavailability). As such, early detection of HACA while on treatment with IFX can guide the physician and/or patient to switch to other anti-TNF drugs or increase the dose of IFX.

[0233] The assays were validated in terms of intra-and inter-assay precision (based on the CV parameter) and susceptibility to interference agents. This analysis is presented below:

Infliximab assay

Parameter	CV%
Intra-assay Precision	2.89
Inter-assay Precision	
Run to Run	4.57
Analyst to Analyst	6.06
Instrument to Instrument	2.73

HACA assay

Parameter	CV%
Intra-assay Precision	3.96
Inter-assay Precision	
Run to Run	4.15
Analyst to Analyst	5.84
Instrument to Instrument	6.88

other serum components has little effect on the mobility shift assay. The mobility shift assay format, unlike ELISA, is not affected by non-specific binding of irrelevant IgGs and detects the IgG4 isotype. Healthy serum samples do not cause mobility shift of the Fl-labeled IFX and 28.2% of the patients treated with IFX were found to have HACA by the assay ~~of the present invention~~. As such, the assay format described herein is very sensitive and can be applied to detect all biologic drugs (*e.g.,* Remicade, Humira, Enbrel and Cimzia) as well as their antibodies (*e.g.,* anti-Remicade, anti-Humira, anti-Enbrel and anti-Cimzia) in patient serum. Notably, since HACA can be detected in the presence of IFX using the mobility shift assay ~~of the invention~~, early detection of HACA while on treatment with IFX can guide the physician and/or patient to switch to other anti-TNF drugs or increase the subsequent dose of IFX.

[0235] We have developed a novel non-radiolabeled, liquid-phase, SE-HPLC assay to measure the IFX and HACA levels in serum samples obtained from patients treated with IFX. The novel assay has high sensitivity, accuracy, and precision, and the results are highly reproducible, which makes this assay suitable for routine testing of a large number of human serum samples. The new assay format, unlike ELISA, eliminates coating of antigens to solid surfaces and is not affected by non-specific binding of irrelevant IgGs. These advantages of the assay format described herein reduce the false negative and false positive results of the test. Advantageously, the assay format ~~of the present invention~~ is very sensitive and can be used to detect all biologic drugs as well as their antibodies present in the serum while the patient is on therapy.

Example 4. Differentiation Between Neutralizing and Non-Neutralizing Human Anti-Chimeric Antibodies (HACA) in Patient Serum Using Novel Mobility Shift Assays.

[0236] This example illustrates novel homogeneous assays for measuring autoantibody (*e.g.,* HACA) concentrations in a patient sample (*e.g.,* serum) and for determining whether such autoantibodies are neutralizing or non-neutralizing autoantibodies using size exclusion chromatography to detect the binding of these autoantibodies to fluorescently labeled anti-TNFα drug in the presence of fluorescently labeled TNFα. These assays are advantageous because they obviate the need for wash steps which remove low affinity HACA, use distinct fluorophores that allow for detection on the visible and/or IR spectra which decreases background and serum interference issues, increase the ability to detect neutralizing or non-neutralizing HACA in patients with a low titer due to the high sensitivity of fluorescent label

detection, and occur as a liquid phase reaction, thereby reducing the chance of any changes in the epitope by attachment to a solid surface such as an ELISA plate.

[0237] In one exemplary methodembodiment, an anti-TNFα drug (*e.g.,* REMICADE™) is labeled with a fluorophore "F1" (*see, e.g.,* Figure 17A), wherein the fluorophore can be detected on either or both the visible and IR spectra. Similarly, TNFα is labeled with a fluorophore "F2" (*see, e.g.,* Figure 17A), wherein the fluorophore can also be detected on either or both the visible and IR spectra, and wherein "F1" and "F2" are different fluorophores. The labeled anti-TNFα drug is incubated with human serum in a liquid phase reaction and the labeled TNFα is added to the reaction to allow the formation of complexes (*i.e.,* immuno-complexes) between the labeled anti-TNFα drug, labeled TNFα, and/or HACA present in the serum. Following incubation, the samples are loaded directly onto a size exclusion column. Binding of both the autoantibody (*e.g.,* HACA) and the labeled TNFα to the labeled anti-TNFα drug results in a leftward shift of the peak (*e.g.,* "Immuno-Complex 1" in Figure 17A) compared to a binary complex between the autoantibody and the labeled anti-TNFα drug (*e.g.,* "Immuno-Complex 2" in Figure 17A), the labeled drug alone, or the labeled TNFα alone. The presence of this ternary complex of autoantibody (*e.g.,* HACA), labeled TNFα, and labeled anti-TNFα drug indicates that the autoantibody present in the serum sample is a non-neutralizing form of the autoantibody (*e.g.,* HACA), such that the autoantibody does not interfere with the binding between the anti-TNFα antibody and TNFα. In one particular method embodiment, as shown in Figure 17A, if non-neutralizing HACA is present in the serum, a shift will be observed for both F1-REMICADE™ and F2-TNFα, resulting in an increase in both the Immuno-Complex 1 and Immuno-Complex 2 peaks and a decrease in the free F1-REMICADE™ and free F2-TNFα peaks. However, the presence of the binary complex between the autoantibody (*e.g.,* HACA) and the labeled anti-TNFα drug (*e.g.,* "Immuno-Complex 2" in Figure 17B) in the absence of the ternary complex of autoantibody (*e.g.,* HACA), labeled TNFα, and labeled anti-TNFα drug indicates that the autoantibody present in the serum sample is a neutralizing form of the autoantibody (*e.g.,* HACA), such that the autoantibody interferes with the binding between the anti-TNFα antibody and TNFα. In one particular method embodiment, as shown in Figure 17B, if neutralizing HACA is present in the serum, a shift will be observed for F1-REMICADE™, resulting in an increase in the Immuno-Complex 2 peak, a decrease in the free F1-REMICADE™ peak, and no change in the free F2-TNFα peak. In certain instances, the presence of neutralizing HACA indicates that the current therapy with REMICADE™ should be switched to another anti-TNFα drug such as HUMIRA™.

[0238] In an alternative method embodiment, the labeled anti-TNFα drug is first incubated with human serum in a liquid phase reaction to allow the formation of complexes (*i.e.,*

immuno-complexes) between the labeled anti-TNFα drug and HACA present in the serum. Following

[0240] **Methods:** The mobility shift assay was based on the shift in retention time of a free antigen versus antigen-antibody immunocomplex on size-exclusion separation. Fluorophore-labeled adalimumab or TNF-α and internal control were mixed with serum samples to measure the mobility shift of free adalimumab and TNF-α in the presence of ADA or drug. The changes in the ratio of free adalimumab or TNF-α to internal control are indicators of immunocomplex formation. Serum concentrations of ADA or adalimumab were determined with standard curves generated by incubating with different concentrations of anti-human IgG antibody or purified adalimumab. Using the mobility shift assay, we measured adalimumab and ADA levels in sera collected from IBD patients treated with adalimumab who had lost response.

[0241] **Results:** Dose-response curves were generated with anti-human IgG antibody for the measurement of mobility shift of labeled adalimumab. The detection limit of the assay was 1 ng of anti-human IgG. Sera from fifty healthy controls were tested for ADA and all of the samples had ADA levels below the detection limit (*i.e.,* no shift of the free labeled-adalimumab). Detection of ADA was also demonstrated in the presence of exogenously added adalimumab. To measure the drug concentration in patients treated with adalimumab, we generated a standard curve with different amounts of adalimumab on the mobility shift of labeled TNF-α, and the detection limit of adalimumab was 10 ng.

[0242] **Conclusions:** The non-radio labeled liquid-phase homogeneous mobility shift assay of the present method ~~invention~~ has been applied to measure ADA and adalimumab levels in serum samples from patients treated with adalimumab. The assay is found to be reproducible with high sensitivity and accuracy, and can be used to evaluate ADA levels in serum samples from patients treated with adalimumab.

Example 6. Analysis of Anti-Drug Antibodies (ADA) to Adalimumab in Patient Serum Using A Novel Proprietary Mobility Shift Assay.

ABSTRACT

[0243] **Background:** Anti-TNF-α drugs such as infliximab (IFX) and adalimumab (ADL) have been shown to be effective in treating inflammatory bowel disease (IBD). However, induction of ADA in the treated patients may reduce the drug's efficacy and/or induce adverse effects. Indeed, ADAs have been found not only in patients treated with IFX, but also in patients treated with ADL. Monitoring of ADA and drug levels in individual patients may help to optimize treatment and dosing of the patient. We have developed a proprietary mobility shift assay to accurately measure in the serum both HACA (Human Anti-Chimeric

of free Fl-labeled ADL to internal control from each sample was used to extrapolate the HAHA concentration from the standard curve. A similar methodology was used to measure ADL levels in patient serum samples with Fl-labeled TNF-α.

RESULTS

[0253] Figure 19 shows the separation of the anti-human IgG bound Fl-ADL complex from the free Fl-ADL due to the mobility shift of the high molecular weight complex. As seen in panels **c** to **h**, the retention time of the fluorescent peak shifted from 10.1 min to 7.3-9.5 min. The more the anti-human IgG is added in the reaction mixture, the less the free ADL remains in the chromatogram and the more the immunocomplex is formed (**h** to **c**). The retention time for the internal control is 13.5 min.

[0254] Figure 20 shows the dose-response curve of the fluorescent peak shift caused by the addition of anti-human IgG. Increasing the concentration of anti-human IgG reduces the ratio of free ADL to internal control due to the formation of the immunocomplex. The assay sensitivity is 10ng/ml of anti-human IgG. The internal control "Fl-BioCyt" corresponds to an Alexa Fluor® 488-biocytin (BioCyt) which combines the green-fluorescent Alexa Fluor® 488 fluorophore with biotin and an aldehyde-fixable primary amine (lysine) (Invitrogen Corp.; Carlsbad, CA).

[0255] Figure 21 shows the separation of the ADL bound TNF-α-Fl complex from the free TNF-α-Fl due to the mobility shift of the high molecular weight complex. As seen in panels **c** and **j**, the retention time of the fluorescent peak shifted from 11.9 min to 6.5- 10.5 min. The more the ADL is added in the reaction mixture, the less the free TNF-α-Fl peak remains in the chromatogram and the more the immuno-complex is formed.

[0256] Figure 22 shows the dose-response curves of the TNF-α-Fl peak shift caused by the addition of ADL. Based on the added ADL, the detection limit is 10 ng/mL of ADL in serum.

[0257] Table 6 shows that serum samples from 100 healthy subjects and 114 IBD patients treated with ADL were analyzed for ADA and ADL levels using the mobility shift assay ~~of the present invention~~. All 100 healthy subject samples had ADA levels below the limit of detection (no shift of the free Fl-ADL), whereas 5 out of the 114 patient samples had an ADA concentration of 0.012 to >20 µg/ml. The mean of ADL levels in 100 healthy subject samples was 0.76±1.0 µg/ml (range 0 to 9.4 µg/ml). The mean of ADL levels in 114 serum samples from patients treated with ADL was 10.8+17.8 µg/ml (range 0 – 139 µg/ml). Four out of five ADA positive samples had undetectable levels of ADL.

[0259] Healthy control serum samples do not cause mobility shift of the Fl-labeled ADL. In a preliminary study, 9.52% of patients with 0.4 μg/ml ADL were found to have ADA in this assay.

Example 7: Determining the Concentration Levels of REMICADE™and Human Anti-Drug Antibodies.

[0260] This example describes a method for determining the levels of Anti-TNFα Drugs, *e.g.* REMICADE™ (infliximab), in a serum sample as well as for determining the levels of a human anti-drug antibody, *e.g.* a human anti-chimeric antibody (HACA) to REMICADE™ (infliximab).

[0261] **Step 1**: **Determining concentration level of REMICADE™ (infliximab) in a sample.**

[0262] ~~In one exemplary embodiment,~~ TNFα ~~is~~ can be labeled with a fluorophore (*e.g.* $Alexa_{647}$), wherein the fluorophore can be detected by, either or both of, the visible and fluorescent spectra. The labeled TNFα is incubated with human serum in a liquid phase reaction to allow the anti-TNFα drug present in the serum to bind. The labeled TNFα can also be incubated with known amounts of the anti-TNFα drug in a liquid phase reaction to create a standard curve. Following incubation, the samples are loaded directly onto a size exclusion column. Binding of the anti-TNFα drug to the labeled TNFα results in a leftward shift of the peak compared to labeled TNFα alone. The concentration of the anti-TNFα drug present in the serum sample can then be compared to the standard curve and controls.

[0263] **SE-HPLC analysis of REMICADE™ (infliximab) levels in patient serum**. Human recombinant TNFα was labeled with a fluorophore, Alexa Fluor® 488, according to the manufactureR's instructions. Labeled TNFα was incubated with different amounts of REMICADE™ or patient serum for one hour at room temperature. Samples of 100 μL volume were analyzed by size-exclusion chromatography on an HPLC system. Fluorescence label detection was used to monitor the free labeled TNFα and the bound labeled TNFα immuno-complex based on their retention times. Serum REMICADE™ levels were calculated from the standard curve.

[0264] The following equations are relevant to this assay:

Equation I: labeled-TNFα + REMICADE™ $\rightarrow$ $(\text{labeled-TNF}\alpha\bullet\text{REMICADE}^{TM})_{complex}$

Equation II: $[\text{REMICADE}^{TM}]_{\text{without-labeled-TNF}\alpha\text{-present}} = [(\text{labeled-TNF}\alpha\bullet\text{REMICADE}^{TM})_{complex}]$

Equation III: [REMICADE^{TM}] = [(labeled-TNFα•REMICADE^{TM})$_{complex}$]/[labeled-TNFα] x [labeled-TNFα]

[0265] In **Step 1**, a known amount of the labeled-TNFα is contacted with a REMICADE^{TM}-containing serum sample. The labeled-TNFα and the REMICADE^{TM} form a complex, (labeled-TNFα•REMICADE^{TM})$_{complex}$, **See Equation I**. Because almost all of the REMICADE^{TM} will form a complex with the labeled-TNFα, the concentration of REMICADE^{TM} present before introduction of the labeled-TNFα is equal to the measured concentration of labeled-TNFα•REMICADE^{TM}$_{complex}$, **See Equation II**. The concentration level of REMICADE^{TM} is calculated by multiplying the ratio of [(label-TNFα•REMICADE^{TM})$_{complex}$]/[labeled-TNFα] by [labeled-TNFα], **See Equation III**. The ratio, [(label-TNFα•REMICADE^{TM})$_{complex}$]/[labeled-TNFα], is obtained by integrating the area-under-the curve for the (label-TNFα•REMICADE^{TM})$_{complex}$ peak, from a plot of signal intensity as a function of elution time from the size exclusion HPLC, and dividing this number by the resultant integration of the area-under-the-curve for the labeled-TNFα peak from the plot. The [labeled-TNFα] is known *a priori*.

[0266] **Step 2**: **Determining level of human anti-chimeric antibody, HACA.**

[0267] ~~In one exemplary embodiment, a~~An anti-TNFα drug, *e.g.,* REMICADE™ can be~~, is~~ labeled with a fluorophore, *e.g.,* Alexa$_{647}$, wherein the fluorophore can be detected by, either or both of, the visible and fluorescent spectra. The labeled anti-TNFα drug is incubated with human serum in a liquid phase reaction to allow any HACA present in the serum to bind. The labeled anti-TNFα drug can also be incubated with known amounts of an anti-IgG antibody or pooled positive patient serum in a liquid phase reaction to create a standard curve. Following incubation, the samples are loaded directly onto a size exclusion column. Binding of the autoantibodies to the labeled anti-TNFα drug results in a leftward shift of the peak compared to labeled drug alone. The concentration of HACA present in the serum sample can then be compared to the standard curve and controls.

[0268] **SE-HPLC analysis of HACA levels in patient serum.** Purified REMICADE^{TM} was labeled with a fluorophore. Labeled REMICADE^{TM} was incubated with different dilutions of pooled HACA-positive serum or diluted patient serum for one hour at room temperature. Samples of 100 µL volume were analyzed by size-exclusion chromatography on an HPLC system. Fluorescence label detection was used to monitor the free labeled REMICADE^{TM} and the bound labeled REMICADE^{TM} immuno-complex based on their

was 30 ng multiplied by 0.25, which is equal to 7.5 ng labeled-REMICADE^{TM}•HACA$_{compelx}$. Using **Equation VIII**, the total amount of labeled-REMICADE^{TM}•HACA$_{complex}$ was 37.5 ng multiplied by 0.25, which is equal to 9.4 ng labeled-REMICADE^{TM}•HACA$_{compelx}$. Using **Equation VI,** the total amount of HACA equals the sum of 9.4 ng and 7.5 ng, which equals 16.9 ng HACA. The 16.9 ng HACA was present in 4 µL of sample. The [HACA] was 16.9 ng/4µL, which equals 4.23 µg/ml. Using **Equation IX**, the effective amount of REMICADE^{TM} is equal to 7.5 µg/ml REMICADE^{TM}, determined from **Step 1**, minus 4.23 µg/ml HACA, determined from **Step 2**. In this exemplary calculation, the effective [REMICADE^{TM}] was equal to 3.27µg/ml.

Example 8: Determining the Concentration Levels of HUMIRA^{TM} and Human Anti-Drug Antibodies.

[0274] This example describes a method for determining the levels of HUMIRA^{TM} in a serum sample as well as for determining the levels of human anti-human antibodies (HAHA).

[0275] **Step 1**: **Determining concentration level of HUMIRA^{TM} in a sample.**

[0276] ~~In one exemplary embodiment,~~ TNFα ~~is~~ can be labeled with a fluorophore (*e.g.* Alexa$_{647}$), wherein the fluorophore can be detected by, either or both of, the visible and fluorescent spectra. The labeled TNFα is incubated with human serum in a liquid phase reaction to allow the anti-TNFα drug present in the serum to bind. The labeled TNFα can also be incubated with known amounts of the anti-TNFα drug in a liquid phase reaction to create a standard curve. Following incubation, the samples are loaded directly onto a size exclusion column. Binding of the anti-TNFα drug to the labeled TNFα results in a leftward shift of the peak compared to labeled TNFα alone. The concentration of the anti-TNFα drug present in the serum sample can then be compared to the standard curve and controls.

[0277] **SE-HPLC analysis of HUMIRA^{TM} levels in patient serum**. Human recombinant TNFα was labeled with a fluorophore, Alexa Fluor® 488, according to the manufacturer's instructions. Labeled TNFα was incubated with different amounts of HUMIRA^{TM} or patient serum for one hour at room temperature. Samples of 100 µL volume were analyzed by size-exclusion chromatography on an HPLC system. Fluorescence label detection was used to monitor the free labeled TNFα and the bound labeled TNFα immuno-complex based on their retention times. Serum HUMIRA^{TM} levels were calculated from the standard curve.

[0278] The following equations are relevant to this assay:

Equation X: labeled-TNFα + HUMIRA^{TM} → (labeled-TNFα•HUMIRA^{TM})$_{complex}$

Equation XI: $[HUMIRA^{TM}] = [(labeled\text{-}TNF\alpha \bullet HUMIRA)_{complex}]$

Equation XII: $[HUMIRA^{TM}] = [(label\text{-}TNF\alpha \bullet HUMIRA^{TM})_{complex}]/[labeled\text{-}TNF\alpha] \times [labeled\text{-}TNF\alpha]$

[0279] In **Step 1**, a known amount of the labeled-TNFα is contacted with a HUMIRA™-containing serum sample. The labeled-TNFα and the HUMIRA™ form a complex, (labeled-TNFα•HUMIRA™)$_{complex}$, **See Equation X**. Because almost all of the HUMIRA™ will form a complex with the labeled-TNFα, the [HUMIRA™] present before introduction of the labeled-TNFα is equal to the measured [(labeled-TNFα•HUMIRA™)$_{complex}$], **See Equation XI**. The [HUMIRA™] is calculated by multiplying the ratio of [(label-TNFα•HUMIRA™)$_{complex}$]/[Labeled-TNFα] by [labeled-TNFα], **See Equation XII**. By integrating the area-under-the-curve for the labeled-TNFα and the area-under-the-curve for the (labeled-TNFα•HUMIRA™)$_{complex}$ and dividing the resultant integration for (labeled-TNFα•HUMIRA™)$_{complex}$ by the resultant integration for the labeled-TNFα, the ratio of [(label-TNFα•HUMIRA™)$_{complex}$] to [labeled-TNFα] is obtained. The [labeled-TNFα] is known *a priori*.

[0280] **Step 2**: **Determining level of human anti-human antibody, *e.g.* HAHA.** ~~In one exemplary embodiment, a~~An anti-TNFα drug, *e.g.,* HUMIRA™, ~~is~~ can be labeled with a fluorophore, *e.g.,* Alexa$_{647}$, wherein the fluorophore can be detected by, either or both of, the visible and fluorescent spectra. The labeled anti-TNFα drug is incubated with human serum in a liquid phase reaction to allow any HAHA present in the serum to bind. The labeled anti-TNFα drug can also be incubated with known amounts of an anti-IgG antibody or pooled positive patient serum in a liquid phase reaction to create a standard curve. Following incubation, the samples are loaded directly onto a size exclusion column. Binding of the autoantibodies to the labeled anti-TNFα drug results in a leftward shift of the peak compared to labeled drug alone. The concentration of HAHA present in the serum sample can then be compared to the standard curve and controls.

[0281] **SE-HPLC analysis of HAHA levels in patient serum.** Purified HUMIRA™ was labeled with a fluorophore. Labeled HUMIRA™ was incubated with different dilutions of pooled HAHA-positive serum or diluted patient serum for one hour at room temperature. Samples of 100 µL volume were analyzed by size-exclusion chromatography on an HPLC system. Fluorescence label detection was used to monitor the free labeled HUMIRA™ and the bound labeled HUMIRA™ immuno-complex based on their retention times. The ratio of

the uncomplexed anti-TNFα drug and then dividing the resultant integration for the complexed anti-TNFα drug by the resultant integration for the uncomplexed anti-TNFα drug.

[0290] ~~In one embodiment, t~~The uncomplexed anti-TNFα drug ~~is~~ can be REMICADE™ having levels between about 0 ng and 100 ng in a sample. The amount of labeled-REMICADE™ is about 37.5 ng.

[0291] By using an internal control, *e.g.* Biocytin-Alexa 488, serum artifacts and variations from one experiment to another experiment can be identified and properly analyzed. The amount of internal control, *e.g.* Biocytin-Alexa 488, is from about 50 to about 200 pg per 100 µL analyzed.

[0292] The ratio of the labeled anti-TNFα drug, *e.g.* REMICADE™ or HUMIRA™, to the labeled internal control is obtained by integrating the the areas-under-the-curve for both the labeled anti-TNFα drug and the labeled internal control and then dividing the resultant integration for the labeled anti-TNFα drug by the resultant integration for the labeled internal control.

[0293] The ratio of [(labeled-anti-TNFα Drug•Autoantibody)$_{complex}$]/[internal control] is obtained by integrating the area-under-the curve for the (labeled-anti-TNFα drug•Autoantibody)$_{complex}$ peak from a plot of signal intensity as a function of elution time from the size exclusion HPLC, and dividing this number by the resultant integration of the area-under-the-curve for the internal control peak from the plot. ~~In some embodiments, t~~The labeled anti-TNFα drug ~~is~~ can be labeled REMICADE™. ~~In some other embodiments, t~~The labeled anti-TNFα drug ~~is~~ can be labeled HUMIRA™.

Example 11: Determining the Ratio of free and complexed labeled TNFα.

[0294] This example describes a method for determining the amount of a complex of labeled-TNFα with either REMICADE™ or HUMIRA™ with reference to an internal standard.

[0295] By using an internal control, *e.g.* Biocytin-Alexa 488, serum artifacts and variations from one experiment to another experiment can be identified and properly analyzed. The amount of internal control, *e.g.* Biocytin-Alexa 488, is from about 1 to about 25 ng per 100 µL analyzed.

[0296] ~~In one embodiment, t~~The uncomplexed labeled TNFα ~~has~~ can have levels between about 50 ng and 150 ng in a sample. In certain instances, the amount of labeled-TNFα is about 100.0 ng.

[0297] Fluorophore (Fl)-labeled TNFα was incubated with patient serum to form the immunocomplex. A Fl-labeled small peptide, *e.g.* Biocytin-Alexa 488, was included as an internal control in each reaction. A standard curve was created by spiking in known concentrations of purified anti-TNFα drug and then extrapolating from the curve to determine the concentration in units of μg/mL.

[0298] The initial ratio of the Labeled-TNFα to the internal control is equal to 100. When the ratio of the Labeled-TNFα to the internal control falls below 95, the labeled-TNFα is inferred to be complexed with an anti-TNFα drug, *e.g.* Remicade™, Humira™. The ratio of the [Labeled-TNFα] to [internal control] is obtained by integrating the areas-under-the-curve for the Labeled-TNFα and for the internal control and then dividing the resultant integration for the Labeled-TNFα by the resultant integration for the internal control.

Example 12: Optimizing Anti-TNFα Drug Therapy by Measuring Anti-TNFα Drug and/or Anti-Drug Antibody (ADA) Levels.

[0299] This example describes methods for optimizing anti-TNFα drug therapy, reducing toxicity associated with anti-TNFα drug therapy, and/or monitoring the efficacy of therapeutic treatment with an anti-TNFα drug by measuring the amount (*e.g.,* concentration level) of anti-TNFα drug (*e.g.,* level of free anti-TNFα therapeutic antibody) and/or anti-drug antibody (ADA) (*e.g.,* level of autoantibody to the anti-TNFα drug) in a sample from a subject receiving anti-TNFα drug therapy. Accordingly, the methods set forth in the present example provide information useful for guiding treatment decisions, *e.g.,* by determining when or how to adjust or modify (*e.g.,* increase or decrease) the subsequent dose of an anti-TNFα drug, by determining when or how to combine an anti-TNFα drug (*e.g.,* at an increased, decreased, or same dose) with one or more immunosuppressive agents such as methotrexate (MTX) or azathioprine, and/or by determining when or how to change the current course of therapy (*e.g.,* switch to a different anti-TNFα drug).

[0300] For purposes of illustration only, the following scenarios provide a demonstration of how the methods ~~of the present invention~~ advantageously enable therapy to be optimized and toxicity (*e.g.,* side-effects) to be minimized or reduced based upon the level of anti-TNFα drug (*e.g.,* level of free anti-TNFα therapeutic antibody) and/or ADA (*e.g.,* level of autoantibody to the anti-TNFα drug) in a sample from a subject receiving anti-TNFα drug therapy. The levels of the anti-TNFα drug and ADA can be measured with the novel assays described herein.

Table 11

Existing Paradigm

HACA	DRUG	Action
LOW	LOW	Increase Dose
MID	LOW	Increase Dose
HIGH	LOW	Switch Therapy
LOW	MID	Continue
MID	MID	**Indeterminate**
HIGH	MID	Switch Therapy
LOW	HIGH	Continue
MID	HIGH	Continue
HIGH	HIGH	Switch Therapy

[0344] This paradigm is confounded, however, by the high variability in drug levels in HACA-indeterminant patients.

[0345] The therapeutic paradigm of the present method ~~invention~~ utilizes a disease activity/severity index derived from an algorithmic-based analysis of one or more biomarkers to select therapy, optimize therapy, reduce toxicity, monitor the efficacy of therapeutic treatment, or a combination thereof, with an anti-TNF drug. In certain aspects, the actions to be taken based on this new paradigm are outline for various illustrative scenarios in the following Table 12:

Table 12. Paradigm of the Present Method ~~Invention~~

HACA	DRUG	Disease Activity Index	Action
LOW	LOW	LOW	Continue
LOW	LOW	MID	Increase Dose
LOW	LOW	HIGH	Increase Dose
LOW	MID	LOW	Continue
LOW	MID	MID	Increase Dose
LOW	MID	HIGH	Increase Dose
LOW	HIGH	LOW	Continue or Decrease Dose to avoid toxicity
LOW	HIGH	MID	Continue
LOW	HIGH	HIGH	Switch Therapy
MID	LOW	LOW	Continue
MID	LOW	MID	Increase Dose
MID	LOW	HIGH	Increase Dose or Change Therapy
MID	MID	LOW	Continue
MID	MID	MID	Continue
MID	MID	HIGH	Switch Therapy
MID	HIGH	LOW	Continue or Decrease Dose to avoid toxicity
MID	HIGH	MID	Continue
MID	HIGH	HIGH	Switch Therapy
HIGH	LOW	LOW	Switch Therapy
HIGH	LOW	MID	Switch Therapy
HIGH	LOW	HIGH	Switch Therapy
HIGH	MID	LOW	Switch Therapy
HIGH	MID	MID	Switch Therapy
HIGH	MID	HIGH	Switch Therapy
HIGH	HIGH	LOW	Switch Therapy
HIGH	HIGH	MID	Switch Therapy
HIGH	HIGH	HIGH	Switch Therapy

real time, liquid phase assay resolves Remicade-TNF complexes from free TNF based on the size of the complexes formed.

[0349] While the current lower limit of quantitation is suitable for the majority of patients, there is a need to increase the sensitivity for use in RA patients (see above). In one aspect, the assay relies on detection of 25ng of TNF-Alexa488 in a 100uL injection on the HPLC size exclusion column. The use of fluorescence as the method of detection provides flexibility for optimization of excitation and emission wavelengths as well as the ability to increase the gain of the photomultiplier tube (PMT). The current settings used for validation of the Remicade assay are:

$$\text{FLD } \lambda_{Ex}=494,\ \lambda_{Em}=519$$

$$\text{PMTGain} = 12$$

These settings were chosen based on published wavelengths for the AlexaFluor 488 group as well as normal PMTGain settings for the Agilent 1200 series FLD. Increasing the PMTGain increases the signal and the noise, but up to a certain factor the increase in signal is higher than the increase in the noise. The step from gain to gain is equal to a factor of 2. The most important parameters to optimize are the excitation and emission wavelengths and while the published maximums are a useful staring point, it is often necessary to optimize them because the excitation depends on the compounds themselves as well as the specific instrument characteristics.

[0350] When detecting low amounts of Remicade, a specific peak reflecting a complex of TNF-Alexa488 and Remicade arises at a retention time of 9.2 minutes. In one aspect, it is important for the height of this peak to be at least 3 times over background and that the calculated serum concentration to over multiple replicates to have a coefficient of variance less than 20%. ~~In certain embodiments, t~~The signal to noise of this specific Remicade-TNFAlexa488 peak to normal human serum background ~~is~~ can thus be the starting point for increasing the sensitivity of the assay.

[0351] To increase the sensitivity, the PMTGain as well as the excitation and emission wavelengths were optimized based on the results of amplification plots and isoabsorbance plots. Remicade was titrated in the presence of dilutions of TNF-Alexa488 at different PMTGain levels ranging from 12-18, using the current excitation and emission wavelengths of 494 and 519 nm, respectively.

[0364] **Conclusions:** Results from this study demonstrated the superiority of the mobility shift assay in measuring HACA and IFX in patient serum samples. This method can also be applied to detect other biopharmaceuticals and ADA in patient serum samples such as those treated with adalimumab.

~~**[0379]** Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, one of skill in the art will appreciate that certain changes and modifications may be practiced within the scope of the appended claims. In addition, each reference provided herein is incorporated by reference in its entirety to the same extent as if each reference was individually incorporated by reference.~~

WHAT IS CLAIMED IS:

1. A method for detecting the presence or level of an autoantibody to an anti-TNF~~α~~alpha drug in a sample without interference from the anti-TNF~~α~~alpha drug in the sample, the method comprising:

(a) contacting the sample with an acid to dissociate preformed complexes of the autoantibody and the anti-TNF~~α~~alpha drug, wherein the sample has or is suspected of having an autoantibody to the anti-TNF~~α~~alpha drug;

(b) contacting the sample with a labeled anti-TNF~~α~~alpha drug following dissociation of the preformed complexes, wherein optionally step (b) further comprises contacting a labeled internal control with the sample;

(c) neutralizing the acid in the sample to form labeled complexes of the labeled anti-TNF~~α~~alpha drug and the autoantibody;

(d) subjecting the labeled complexes to size exclusion chromatography to separate the labeled complexes; and

(e) detecting the labeled complexes, thereby detecting the presence or level of the autoantibody without interference from the anti-TNF~~α~~alpha drug in the sample.

~~2. The method of claim 1, wherein the anti-TNFα drug is selected from the group consisting of REMICADE™ (infliximab), ENBREL™ (etanercept), HUMIRA™ (adalimumab), CIMZIA® (certolizumab pegol), SIMPONI® (golimumab; CNTO 148), and combinations thereof.~~

~~3. The method of claim 1 or 2, wherein the autoantibody to the anti-TNFα drug is selected from the group consisting of a human anti-chimeric antibody (HACA), a human anti-humanized antibody (HAHA), a human anti-mouse antibody (HAMA), and combinations thereof.~~

~~4. The method of any one of claims 1 to 3, wherein the acid comprises an organic acid, an inorganic acid, or mixtures thereof.~~

~~5. The method of claim 4, wherein the organic acid comprises citric acid.~~

~~6~~2. The method of ~~any one of claims 1 to 5~~claim 1, wherein the sample is contacted with an acid at a concentration of from about 0.1M to about 5M.

~~7~~3. The method of any one of claims 1 to ~~6~~2, wherein the acid is neutralized by adding one or more neutralizing agents to the sample.

~~8. The method of any one of claims 1 to 7, wherein step (b) further comprises contacting a labeled internal control with the sample.~~

~~9. The method of any one of claims 1 to 8, wherein the presence or level of the autoantibody is detected in the presence of a high level of the anti-TNFα drug.~~

~~10. The method of claim 9, wherein the high level of the anti-TNFα drug corresponds to an anti-TNFα drug level of from about 10 to about 100 µg/mL.~~

~~11. The method of any one of claims 1 to 10, wherein the size exclusion chromatography is size exclusion high performance liquid chromatography (SE-HPLC).~~

~~12. The method of any one of claims 1 to 11, wherein the sample is serum.~~

~~13~~4. The method of any one of claims 1 to ~~12~~3, wherein the sample is obtained from a subject receiving therapy with the anti-TNF~~α~~alpha drug.

~~14~~5. The method of any one of claims 1 to ~~13~~4, wherein the complexes are eluted first, followed by free labeled anti-TNF~~α~~alpha drug.

~~15~~6. The method of any one of claims 1 to ~~14~~5, wherein the anti-TNF~~α~~alpha drug is labeled with a fluorophore or a fluorescent dye.

~~16~~7. A method for optimizing therapy and/or reducing toxicity to an anti-TNF~~α~~alpha drug in a subject receiving a course of therapy with the anti-TNF~~α~~alpha drug, the method comprising:

(a) detecting the presence or level of an autoantibody to the anti-TNF~~α~~alpha drug in a sample from the subject without interference from the anti-TNF~~α~~alpha drug in the sample, the method comprising:

(i) contacting the sample with an acid to dissociate preformed complexes of the autoantibody and the anti-TNF~~α~~alpha drug, wherein the sample has or is suspected of having an autoantibody to the anti-TNF~~α~~alpha drug;

(ii) contacting the sample with a labeled anti-TNF~~α~~alpha drug following dissociation of the preformed complexes;

(iii) neutralizing the acid in the sample to form labeled complexes of the labeled anti-TNF~~α~~alpha drug and the autoantibody;

(iv) subjecting the labeled complexes to size exclusion chromatography to separate the labeled complexes; and

(v) detecting the labeled complexes to thereby detect the presence or level of the autoantibody without interference from the anti-TNF~~α~~alpha drug in the sample; and

(b) determining a subsequent dose of the course of therapy for the subject or whether a different course of therapy should be administered to the subject based upon the presence or level of the autoantibody,

thereby optimizing therapy and/or reducing toxicity to the anti-TNF~~α~~alpha drug.

~~17. The method of claim 16, wherein the anti-TNFα drug is selected from the group consisting of REMICADE™ (infliximab), ENBREL™ (etanercept), HUMIRA™ (adalimumab), CIMZIA® (certolizumab pegol), SIMPONI® (golimumab; CNTO 148), and combinations thereof.~~

~~18. The method of claim 16 or 17, wherein the autoantibody to the anti-TNFα drug is selected from the group consisting of a human anti-chimeric antibody (HACA), a human anti-humanized antibody (HAHA), a human anti-mouse antibody (HAMA), and combinations thereof.~~

~~19~~8. The method of ~~any one of claims 16 to 18~~claim 7, wherein the subsequent dose of the course of therapy is increased, decreased, or maintained based upon the presence or level of the autoantibody.

~~20. The method of claim 19, wherein the subsequent dose of the course of therapy is decreased when a high level of the autoantibody is detected in the sample.~~

~~21. The method of any one of claims 16 to 18, wherein the different course of therapy comprises a different anti-TNFα drug.~~

~~22. The method of any one of claims 16 to 18, wherein the different course of therapy comprises the current course of therapy along with an immunosuppressive agent.~~

~~23. The method of any one of claims 16 to 18, wherein the different course of therapy comprises switching to a course of therapy that is not an anti-TNFα drug.~~

~~24. The method of any one of claims to 21 to 23, wherein the different course of therapy is administered when a high level of the autoantibody is detected in the sample.~~

9. The method of claims 7 or 8, wherein the different course of therapy comprises a different anti-TNFalpha drug, or wherein the different course of therapy comprises the current course of therapy along with an immunosuppressive agent, or wherein the different course of therapy comprises switching to a course of therapy that is not an anti-TNFalpha drug.

10. The method of any of claims 1 to 9, wherein the anti-TNFalpha drug is selected from the group consisting of infliximab, etanercept, adalimumab, certolizumab pegol, golimumab, CNTO 148, and combinations thereof.

11. The method of any of claims 1 to 10, wherein the autoantibody to the anti-TNFalpha drug is selected from the group consisting of a human anti-chimeric antibody (HACA), a human anti-humanized antibody (HAHA), a human anti-mouse antibody (HAMA), and combinations thereof.

~~25~~12. The method of any one of claims ~~16 to 24~~1 to 11, wherein the acid comprises an organic acid, an inorganic acid, or mixtures thereof, wherein optionally the organic acid comprises citric acid.

~~26~~13. The method of any one of claims ~~16 to 25~~1 to 12, wherein the presence or level of the autoantibody is detected in the presence of a high level of the anti-TNF~~α~~alpha drug, wherein optionally the high level of the anti-TNFalpha drug corresponds to an anti-TNFalpha drug level of from 10 to 100 µg/ml.

~~27~~14. The method of any one of claims ~~16 to 26~~1 to 13, wherein the size exclusion chromatography is size exclusion-high performance liquid chromatography (SE-HPLC).

~~28~~15. The method of any one of claims ~~16 to 27~~1 to 14, wherein the sample is serum.