

METHODS FOR DETERMINING ANTI-DRUG ANTIBODY ISOTYPES

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims priority to U.S. Provisional Application No. 61/394,269,
5 filed October 18, 2010, the disclosure of which is hereby incorporated by reference in its
entirety for all purposes.

BACKGROUND OF THE INVENTION

[0002] Autoimmune disorders are a significant and widespread medical problem. For
example, rheumatoid arthritis (RA) is an autoimmune disease affecting more than two
10 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
15 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.

20 [0003] 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
25 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-
30 effects. Thus, additional therapies for rheumatoid arthritis and other autoimmune disorders
have been sought.

[0004] Tumor necrosis factor alpha (TNF α) is a cytokine produced by numerous cell types, including monocytes and macrophages, that was originally identified based on its ability to induce the necrosis of certain mouse tumors. Subsequently, a factor termed cachectin,

5 associated with cachexia, was shown to be identical to TNF α . TNF α has been implicated in the pathophysiology of a variety of other human diseases and disorders, including shock, sepsis, infections, autoimmune diseases, RA, Crohn's disease, transplant rejection and graft-versus-host disease.

[0005] Because of the harmful role of human TNF α (hTNF α) in a variety of human
10 disorders, therapeutic strategies have been designed to inhibit or counteract hTNF α activity. In particular, antibodies that bind to, and neutralize, hTNF α have been sought as a means to inhibit hTNF α activity. Some of the earliest of such antibodies were mouse monoclonal antibodies (mAbs) secreted by hybridomas prepared from lymphocytes of mice immunized with hTNF α (*see, e.g.*, U.S. Pat. No. 5,231,024). While these mouse anti-
15 hTNF α antibodies often displayed high affinity for hTNF α and were able to neutralize hTNF α activity, their use *in vivo* has been limited by problems associated with the administration of mouse antibodies to humans, such as a short serum half-life, an inability to trigger certain human effector functions, and elicitation of an unwanted immune response against the mouse antibody in a human (the "human anti-mouse antibody"
20 (HAMA) reaction).

[0006] More recently, biological therapies have been applied to the treatment of autoimmune disorders such as rheumatoid arthritis. For example, four TNF α inhibitors, REMICADE™ (infliximab), a chimeric anti-TNF α mAb, ENBREL™ (etanercept), a TNFR-Ig Fc fusion protein, HUMIRA™ (adalimumab), a human anti-TNF α mAb, and
25 CIMZIA® (certolizumab pegol), a PEGylated Fab fragment, have been approved by the FDA for treatment of rheumatoid arthritis. CIMZIA® is also used for the treatment of moderate to severe Crohn's disease (CD). While such biologic therapies have demonstrated success in the treatment of rheumatoid arthritis and other autoimmune disorders such as CD, not all subjects treated respond, or respond well, to such therapy.

30 **[0007]** Moreover, administration of TNF α inhibitors can induce an immune response to the drug and lead to the production of anti-drug antibodies (ADA) such as human anti-chimeric antibodies (HACA), human anti-humanized antibodies (HAHA), and human anti-

mouse antibodies (HAMA). Such HACA, HAHA, or HAMA immune responses can be associated with hypersensitive reactions and dramatic changes in pharmacokinetics and biodistribution of the immunotherapeutic TNF α inhibitor that preclude further treatment with the drug. In addition, the presence of particular isotypes of HACA, HAHA, or
5 HAMA is associated with different clinical outcomes in subjects receiving anti-TNF α therapy.

[0008] Accordingly, there is a need in the art for assays to detect the presence or level of a specific ADA isotype or a particular combination of ADA isotypes in a sample. There is
10 also a need in the art for methods to select an appropriate course of anti-TNF α therapy, to monitor anti-TNF α therapy, and/or to guide treatment decisions. The present invention satisfies these needs and provides related advantages as well.

BRIEF SUMMARY OF THE INVENTION

[0009] The present invention provides assay methods for the determination of one or
15 more anti-drug antibody (ADA) isotypes in a sample. As a non-limiting example, the assays of the present invention are particularly useful for determining different ADA isotypes in samples from ADA-positive patients receiving an anti-TNF α drug such as REMICADE™ (infliximab) or HUMIRA™ (adalimumab). The present invention also provides methods for optimizing therapy and/or reducing toxicity in subjects receiving
20 TNF α inhibitors for the treatment of TNF α -mediated disease or disorders.

[0010] In one aspect, the present invention provides a method for detecting the presence (or absence) or level of at least one isotype (*e.g.*, a plurality of isotypes) of an autoantibody to an anti-TNF α drug in a sample, the method comprising:

- (a) contacting a labeled anti-TNF α drug with a sample having or suspected of
25 having at least one isotype of an autoantibody to the anti-TNF α drug to form labeled complexes between the labeled anti-TNF α drug and each autoantibody isotype;
- (b) subjecting the labeled complexes to size exclusion chromatography to separate the labeled complexes having different autoantibody isotypes from
30 each other and/or from free labeled anti-TNF α drug; and

- (c) detecting the labeled complexes, thereby detecting the presence (or absence) or level of at least one isotype of the autoantibody to the anti-TNF α drug.

[0011] In some embodiments, the anti-TNF α drug is selected from the group consisting of REMICADETM (infliximab), HUMIRATM (adalimumab), ENBRELTM (etanercept), CIMZIA[®] (certolizumab pegol), and combinations thereof. In other embodiments, the autoantibody to the anti-TNF α drug (*e.g.*, anti-drug antibody or “ADA”) 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.

10 [0012] In certain embodiments, the at least one isotype comprises a plurality of at least two, three, four, five, or more isotypes. In other embodiments, the at least one isotype is selected

from the group consisting of IgA, IgD, IgE, IgG, and IgM isotypes, subclasses thereof, and combinations thereof. In some instances, the sample is whole blood, serum, or plasma.

15 [0013] In some embodiments, the autoantibody to the anti-TNF α drug is HACA and the sample is obtained from a subject on REMICADETM (infliximab) therapy. In certain other embodiments, the autoantibody to the anti-TNF α drug is HAHA and the sample is obtained from a subject on HUMIRATM (adalimumab) therapy.

[0014] In some instances, the labeled anti-TNF α drug is a fluorophore-labeled anti-TNF α drug. In particular embodiments, the labeled complexes are detected using fluorescence label detection. In certain embodiments, each autoantibody isotype is characterized or identified or detected by its retention time. In other embodiments, the size exclusion chromatography is size exclusion-high performance liquid chromatography (SE-HPLC).

25 [0015] In one related aspect, the present invention provides a method for detecting the presence (or absence) or level of at least one isotype (*e.g.*, a plurality of isotypes) of an autoantibody to an anti-TNF α drug in a sample, the method comprising:

- (a) contacting a labeled anti-TNF α drug and one or a plurality of labeled anti-Ig antibodies specific for different antibody isotypes with a sample having or suspected of having at least one isotype of an autoantibody to the anti-TNF α drug to form labeled complexes between the labeled anti-TNF α drug,
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the labeled anti-Ig antibodies, and each autoantibody isotype, wherein the labeled anti-TNF α drug and the labeled anti-Ig antibodies comprise different labels;

- 5 (b) subjecting the labeled complexes to size exclusion chromatography to separate the labeled complexes having different autoantibody isotypes from each other, from free labeled anti-TNF α drug, and/or from free labeled anti-Ig antibodies; and
- (c) detecting the labeled complexes, thereby detecting the presence (or absence) or level of at least one isotype of the autoantibody to the anti-TNF α drug.
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[0016] In some embodiments, the anti-TNF α drug is selected from the group consisting of REMICADETM (infliximab), HUMIRATM (adalimumab), ENBRELTM (etanercept), CIMZIA[®] (certolizumab pegol), and combinations thereof. In other embodiments, the autoantibody to the anti-TNF α drug (*e.g.*, anti-drug antibody or “ADA”) 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.

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[0017] In particular embodiments, the labeled anti-TNF α drug and the one or plurality of labeled anti-Ig antibodies bind to different epitopes of the at least one isotype. As one non-limiting example, the labeled anti-TNF α drug and labeled anti-Ig isotype-specific antibody bind to different epitopes of a particular autoantibody isotype.

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[0018] In certain embodiments, the at least one isotype comprises a plurality of at least two, three, four, five, or more isotypes. In other embodiments, the at least one isotype is selected from the group consisting of IgA, IgD, IgE, IgG, and IgM isotypes, subclasses thereof, and combinations thereof.

25 **[0019]** In some embodiments, the plurality of labeled anti-Ig antibodies comprises at least two, three, four, five, or more labeled anti-Ig antibodies specific for different antibody isotypes. In other embodiments, the one or plurality of labeled anti-Ig antibodies is selected from the group consisting of antibodies specific for one or more of IgA, IgD, IgE, IgG, and IgM isotypes, subclasses thereof, and combinations thereof. In some instances, the sample is whole blood, serum, or plasma.

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[0020] In some embodiments, the autoantibody to the anti-TNF α drug is HACA and the sample is obtained from a subject on REMICADETM (infliximab) therapy. In certain other

embodiments, the autoantibody to the anti-TNF α drug is HAHA and the sample is obtained from a subject on HUMIRATM (adalimumab) therapy.

[0021] In some instances, the labeled anti-TNF α drug is a fluorophore-labeled anti-TNF α drug. In certain other instances, the labeled anti-Ig antibodies are fluorophore-labeled anti-Ig antibodies. The plurality of labeled anti-Ig antibodies specific for different antibody isotypes may each comprise the same label or different labels. In one non-limiting example, the plurality of labeled anti-Ig isotype-specific antibodies is each labeled with Alexa-532 and the labeled anti-TNF α drug is labeled with Alexa-488.

[0022] In some embodiments, the labeled complexes are detected using fluorescence label detection. In particular embodiments, the labeled complexes are detected upon a signal that is generated by the proximity binding of both the labeled anti-TNF α drug and the labeled anti-Ig antibodies to the autoantibody isotype. In certain instances, the signal comprises a fluorescent signal that is detected by fluorescence resonance energy transfer (FRET). In other embodiments, the size exclusion chromatography is size exclusion-high performance liquid chromatography (SE-HPLC). In certain embodiments, each autoantibody isotype is characterized or identified or detected by its retention time.

[0023] In yet another 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 TNF α -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); 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 statistical algorithm applied in step (b).

[0024] As a non-limiting example, the presence, level, or genotype of one, two, three, four, five, or all six of the following classes of biochemical markers, serological markers, and/or genetic markers can be detected, measured, or determined in a patient sample (*e.g.*, a serum sample from a patient on anti-TNF drug therapy):

- (1) anti-TNF drug levels (*e.g.*, levels of free anti-TNF α therapeutic antibody);
- (2) anti-drug antibody (ADA) levels (*e.g.*, level of autoantibody to the anti-TNF drug);

- (3) TNF α levels;
- (4) levels of one, two, three, four, five, six, seven, or more additional cytokines (*e.g.*, IL-6, IL-1 β , IFN- γ , IL-10, *etc.*) and/or markers for other mechanisms of inflammation (*e.g.*, inflammatory markers such as CRP, SAA, ICAM-1, and/or VCAM-1);
- (5) the presence or absence of one or more mutations in one or more genetic markers such as inflammatory pathway genes, *e.g.*, the presence or absence of variant alleles (*e.g.*, SNPs) in one or more inflammatory markers such as, *e.g.*, NOD2/CARD15 (*e.g.*, SNP 8, SNP 12, and/or SNP 13 described in US Patent No. 7,592,437), ATG16L1 (*e.g.*, the rs2241880 (T300A) SNP described in Lakatos *et al.*, *Digestive and Liver Disease*, 40 (2008) 867-873), IL23R (*e.g.*, the rs11209026 (R381Q) SNP described in Lakatos *et al.*), the human leukocyte antigen (HLA) genes and/or cytokine genes described in, *e.g.*, Gasche *et al.* (*Eur. J. Gastroenterology & Hepatology*, (2003) 15:599-606), and the DLG5 and/or OCTN genes from the IBD5 locus;
- (6) levels of one or more of the above biochemical markers and/or serological markers at multiple time points (*e.g.*, at 28 weeks, 60 weeks, *etc.*); and
- (7) combinations thereof.

[0025] In particular embodiments, a single statistical algorithm or a combination of two or more statistical algorithms can then be applied to the presence, level (concentration level), or genotype of the one or more (*e.g.*, a combination of two, three, four, five, six, seven, or more) markers detected, measured, or determined in the sample to thereby optimize therapy, reduce toxicity, and/or monitor the efficacy of therapeutic treatment with the anti-TNF drug. As such, the methods of the present invention find utility in determining patient management by determining patient immune status.

[0026] Methods for detecting anti-TNF drugs (*e.g.*, anti-TNF α antibodies) and anti-drug antibodies (ADA) such as HACA and HAHA 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.

[0027] 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

- [0028] **Figure 1** shows an exemplary embodiment of the assays of the present invention wherein size exclusion HPLC is used to detect the binding between TNF α -Alexa₆₄₇ and HUMIRATM.
- 5 [0029] **Figure 2** shows dose response curves of HUMIRATM binding to TNF α -Alexa₆₄₇.
- [0030] **Figure 3** shows a current ELISA-based method for measuring HACA levels, known as the bridging assay.
- [0031] **Figure 4** illustrates an exemplary outline of the autoantibody detection assays of the present invention for measuring the concentrations of HACA/HAHA generated against
- 10 REMICADETM.
- [0032] **Figure 5** shows a dose response analysis of anti-human IgG antibody binding to REMICADETM-Alexa₆₄₇.
- [0033] **Figure 6** shows a second dose response analysis of anti-human IgG antibody binding to REMICADETM-Alexa₆₄₇.
- 15 [0034] **Figure 7** shows dose response curves of anti-human IgG antibody binding to REMICADETM-Alexa₆₄₇.
- [0035] **Figure 8** shows REMICADETM-Alexa₆₄₇ immunocomplex formation in normal human serum and HACA positive serum.
- [0036] **Figure 9** provides a summary of HACA measurements from 20 patient serum
- 20 samples that were performed using the bridging assay or the mobility shift assay of the present invention.
- [0037] **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.
- [0038] **Figure 11** shows SE-HPLC profiles of fluorophore (Fl)-labeled IFX incubated
- 25 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.
- [0039] **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-FI. The higher the dilution (less HACA) the more free IFX-FI was found in the SE-HPLC analysis. (B) Increasing dilutions of HACA-positive serum were incubated with 37.5 ng of IFX-FI. The higher the dilution (less HACA) the less HACA bound IFX-FI was found in the SE-HPLC analysis.

[0040] Figure 13 shows SE-HPLC profiles of TNF α -FI 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 TNF α peak to the higher molecular mass eluting positions.

[0041] Figure 14 shows dose-response curves of the bound and free TNF α 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 TNF α while increasing the percentage of bound TNF α .

[0042] 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.

[0043] 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.

[0044] 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.

[0045] Figure 18 shows an alternative embodiment of the assays of the present invention to detect the presence of neutralizing autoantibodies such as HACA.

[0046] Figure 19 shows mobility shift profiles of FI-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 FI-ADL peak (FA) to the higher molecular mass eluting positions, C1 and C2, while the internal control (IC) did not change.

[0047] Figure 20 shows a dose-response curve of anti-human IgG on the shift of free FI-ADL. Increasing amounts of anti-human IgG were incubated with 37.5 ng of FI-ADL and internal control. The more the antibody was added to the reaction mixture the lower the ratio of free FI-ADL to internal control.

- [0048]** **Figure 21** shows mobility shift profiles of FI-labeled TNF- α incubated with normal human serum (NHS) in the presence of different amounts of ADL. $E_x = 494$ nm; $E_m = 519$ nm. The addition of increasing amounts of ADL to the incubation mixture dose-dependently shifted the free TNF-FI peak (FT) to the higher molecular mass eluting positions, while the internal control (IC) peak did not change.
- [0049]** **Figure 22** shows a dose-response curve of ADL on the shift of free TNF- α -FI. Increasing amounts of ADL were incubated with 100 ng of TNF- α -FI and internal control. The more the antibody ADL was added to the reaction mixture the lower the ratio of free TNF- α -FI to internal control.
- 10 **[0050]** **Figure 23** shows the elution times of different ADA isotypes in HACA-positive patient serum.
- [0051]** **Figure 24** shows a plot of the mean of the 100 control samples used in the study described in Example 8.
- [0052]** **Figure 25** shows a plot of the mean of the 100 HACA-positive samples used in
15 the study described in Example 8.
- [0053]** **Figure 26** illustrates a side-by-side comparison of the Primary Peak signals (*i.e.*, corresponding to labeled Remicade signal intensity) for the 100 control samples and the 100 HACA-positive samples used in the study described in Example 8.
- [0054]** **Figure 27** shows a Receiver Operating Characteristic (ROC) curve for the
20 Primary Peak data from Figure 26. The Area Under the Curve (AUC) was 0.986.
- [0055]** **Figure 28** shows a plot of Non-primary peaks on the X-axis (which correspond to the sum of the IgG, IgA, and IgM peaks) versus the Primary Peak on the Y-axis for the 100 HACA-positive samples used in the study described in Example 8.
- [0056]** **Figure 29** shows a plot of the IgG versus IgA versus IgM peaks for all 200
25 samples used in the study described in Example 8.
- [0057]** **Figure 30** shows a schematic of a fluorescence resonance energy transfer (FRET) autoantibody isotyping assay format of the present invention.
- [0058]** **Figure 31** shows the results of a FRET-based autoantibody isotyping assay of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

I. Introduction

[0059] TNF α has been implicated in inflammatory diseases, autoimmune diseases, viral, bacterial and parasitic infections, malignancies, and/or neurodegenerative diseases and is a useful target for specific biological therapy in diseases such as rheumatoid arthritis (RA) and Crohn's disease (CD). TNF α inhibitors such as anti-TNF α antibodies are an important class of therapeutics. However, administration of TNF α inhibitors can induce an immune response to the drug and lead to the production of anti-drug antibodies (ADA), thereby precluding further treatment with the drug. In addition, the presence of particular ADA isotypes can be associated with different clinical outcomes in subjects receiving anti-TNF α therapy.

[0060] The present invention is based in part on the discovery that a homogeneous mobility shift assay using size exclusion chromatography is particularly advantageous for measuring the presence or level of one or more ADA isotypes in a sample. As a non-limiting example, the assays of the present invention are particularly useful for determining different ADA isotypes in samples from ADA-positive patients receiving an anti-TNF α drug such as REMICADE™ (infliximab) or HUMIRA™ (adalimumab).

[0061] In particular, the present invention provides "mix and read" ADA isotyping assays that do not require any wash steps. As a result, complexed and uncomplexed reagents are easily separated from each other. In addition, any potential interference from the free anti-TNF α drug is minimized using the assays of the present invention. In contrast, a typical ELISA for measuring autoantibody levels cannot be performed until the TNF α inhibitor is eliminated from the body, which can take up to 3 months. Moreover, the present invention is generally applicable to a wide variety of anti-TNF α therapeutics in addition to anti-TNF α antibodies. The assays of the present invention are also advantageous because they avoid the attachment of antigens to solid surfaces, eliminate the non-specific binding of irrelevant IgGs, detect antibodies with weak affinities, and exhibit increased sensitivity and specificity over currently available detection methods such as enzyme immunoassays.

[0062] The importance of measuring serum concentrations of anti-TNF α biologics (*e.g.*, anti-TNF α antibodies) as well autoantibodies generated against them (*e.g.*, ADA isotypes) is illustrated by the fact that the FDA requires pharmacokinetic and tolerability (*e.g.*,

immune response) studies to be performed during clinical trials. The present invention also finds utility in monitoring patients receiving these drugs to make sure they are getting the right dose, that the drug isn't being cleared from the body too quickly, and that they are not developing an immune response against the drug. Furthermore, the present invention
5 is useful in guiding the switch between different drugs due to failure with the initial drug.

[0063] The present invention also provides methods for optimizing therapy and/or reducing toxicity in subjects receiving TNF α inhibitors for the treatment of TNF α -mediated diseases or disorders. Moreover, the present invention is particularly advantageous because it addresses and overcomes current limitations associated with the
10 administration of anti-TNF drugs such as infliximab or adalimumab, in part, by providing information useful for guiding treatment decisions for those patients receiving or about to receive anti-TNF drug therapy.

II. Definitions

[0064] As used herein, the following terms have the meanings ascribed to them unless
15 specified otherwise.

[0065] The term "TNF α " as used herein 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 TNF α is described further in, for example, Jones *et al.* (1989) *Nature*,
20 338:225-228. The term TNF α is intended to include human TNF α , a recombinant human TNF α (rhTNF α), or a polypeptide having at least about 80% identity to the human TNF α protein. Human TNF α consists of a 35 amino acid (aa) cytoplasmic domain, a 21 aa transmembrane segment, and a 177 aa extracellular domain (ECD) (Pennica *et al.* (1984) *Nature* 312:724). Within the ECD, human TNF α shares 97% aa sequence identity with
25 rhesus and 71% to 92% with bovine, canine, cotton rat, equine, feline, mouse, porcine, and rat TNF α . TNF α can be prepared by standard recombinant expression methods or purchased commercially (*e.g.*, R & D Systems, Catalog No. 210-TA, Minneapolis, Minn.).

[0066] The term "TNF α inhibitor" or "TNF α drug" is intended to encompass agents including proteins, antibodies, antibody fragments, fusion proteins (*e.g.*, Ig fusion proteins
30 or Fc fusion proteins), multivalent binding proteins (*e.g.*, DVD Ig), small molecule TNF α antagonists and similar naturally- or nonnaturally-occurring molecules, and/or recombinant and/or engineered forms thereof, that, directly or indirectly, inhibits TNF α activity, such as

by inhibiting interaction of TNF α with a cell surface receptor for TNF α , inhibiting TNF α protein production, inhibiting TNF α gene expression, inhibiting TNF α secretion from cells, inhibiting TNF α receptor signaling or any other means resulting in decreased TNF α activity in a subject. The term “TNF α inhibitor” or “TNF α drug” preferably includes
5 agents which interfere with TNF α activity. Examples of TNF α drugs include etanercept (ENBREL™, Amgen), infliximab (REMICADE™, Johnson and Johnson), human anti-TNF monoclonal antibody adalimumab (D2E7/HUMIRA™, Abbott Laboratories), CDP 571 (Celltech), and CDP 870 (Celltech), as well as other compounds which inhibit TNF α activity, such that when administered to a subject suffering from or at risk of suffering
10 from a disorder in which TNF α activity is detrimental (*e.g.*, RA), the disorder is treated.

[0067] The term “immunoglobulin isotype” or “antibody isotype” includes any member of a family of related antibodies comprising genetic variations and/or differences in the constant regions of the heavy and/or light chains. Non-limiting examples of antibody isotypes include (1) heavy chain isotypes such as α (*e.g.*, IgA or a subclass thereof such as
15 IgA1 and/or IgA2); δ (*e.g.*, IgD); γ (*e.g.*, IgG or a subclass thereof such as IgG1, IgG2, IgG3, and/or IgG4); ϵ (*e.g.*, IgE); μ (*e.g.*, IgM); and (2) light chain isotypes such as κ and λ .

[0068] The term “size exclusion chromatography” (SEC) is intended to include a chromatographic method in which molecules in solution are separated based on their size
20 and/or hydrodynamic volume. It is applied to large molecules or macromolecular complexes such as proteins and their conjugates. Typically, when an aqueous solution is used to transport the sample through the column, the technique is known as gel filtration chromatography.

[0069] The terms “complex,” “immuno-complex,” “conjugate,” and “immunoconjugate”
25 include, but are not limited to, TNF α bound (*e.g.*, by non-covalent means) to an anti-TNF α drug, an anti-TNF α drug bound (*e.g.*, by non-covalent means) to an autoantibody against the anti-TNF α drug, an anti-TNF α drug bound (*e.g.*, by non-covalent means) to both TNF α and an autoantibody against the anti-TNF α drug, an anti-TNF α drug bound (*e.g.*, by non-covalent means) to an isotype of an autoantibody against the anti-TNF α drug, and an anti-
30 TNF α drug and an anti-Ig isotype-specific antibody bound (*e.g.*, by non-covalent means) to an isotype of an autoantibody against the anti-TNF α drug.

[0070] As used herein, an entity that is modified by the term “labeled” includes any entity, molecule, protein, enzyme, antibody, antibody fragment, cytokine, or related species that is conjugated with another molecule or chemical entity that is empirically detectable. Chemical species suitable as labels for labeled-entities include, but are not limited to, fluorescent dyes (*e.g.* Alexa Fluor[®] dyes such as Alexa Fluor[®] 488, 532, or 647); quantum dots; optical dyes; luminescent dyes; and radionuclides, *e.g.* ¹²⁵I.

[0071] The phrase “fluorescence label detection” includes a means for detecting a fluorescent label. Means for detection include, but are not limited to, a spectrometer, a fluorimeter, a photometer, a detection device commonly incorporated with a chromatography instrument such as, but not limited to, a size exclusion- high performance liquid chromatography, such as, but not limited to, an Agilent-1200 HPLC System.

[0072] The term “optimizing therapy” or “optimize therapy” includes optimizing the dose (*e.g.*, the effective amount or level) and/or the type of a particular therapy. For example, optimizing the dose of an anti-TNF α drug includes increasing or decreasing the amount of the anti-TNF α drug subsequently administered to a subject. In certain instances, optimizing the type of an anti-TNF α drug includes changing the administered anti-TNF α drug from one drug to a different drug (*e.g.*, a different anti-TNF α drug). In certain other instances, optimizing therapy may include co-administering a dose of an anti-TNF α drug (*e.g.*, at an increased, decreased, or same dose as the previous dose) in combination with an immunosuppressive drug.

[0073] The term “course of therapy” includes any therapeutic approach taken to relieve or prevent one or more symptoms associated with a TNF α -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 TNF α -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 TNF α , anti-TNF α drug, and/or anti-drug antibody using the methods of the present invention.

[0074] The term “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 suitable immunosuppressive agents 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.

[0075] 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.

[0076] The term “sample” includes any biological specimen obtained from an individual. Suitable samples for use in the present invention include, without limitation, whole blood, plasma, serum, saliva, urine, stool, tears, any other bodily fluid, tissue samples (*e.g.*, biopsy), and cellular extracts thereof (*e.g.*, red blood cellular extract). In a preferred embodiment, the sample is a serum sample. One skilled in the art will appreciate that samples such as serum samples can be diluted prior to the analysis. In certain instances, the term “sample” includes, but is not limited to blood, body tissue, blood serum, lymph fluid, lymph node tissue, spleen tissue, bone marrow, or an immunoglobulin enriched fraction derived from one or more of these tissues. In certain other instances, the term “sample” includes blood serum or is an immunoglobulin enriched fraction derived from blood serum or blood. In certain instances, the term “sample” includes a bodily fluid.

[0077] The term “predicting responsiveness to a TNF α inhibitor” is intended to refer to an ability to assess the likelihood that treatment of a subject with a TNF α inhibitor 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 TNF α

inhibitors are biologic agents that have been approved by the FDA for use in humans in the treatment of rheumatoid arthritis (RA), which agents are described herein and include, but are not limited to, adalimumab (HUMIRA™), infliximab (REMICADE™) and etanercept (ENBREL™).

- 5 [0078] 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.

III. Description of the Embodiments

[0079] The present invention provides assay methods for the determination of one or
10 more anti-drug antibody (ADA) isotypes in a sample. As a non-limiting example, the assays of the present invention are particularly useful for determining different ADA isotypes in samples from ADA-positive patients receiving an anti-TNF α drug such as REMICADE™ (infliximab) or HUMIRA™ (adalimumab). The present invention also provides methods for optimizing therapy and/or reducing toxicity in subjects receiving
15 TNF α inhibitors for the treatment of TNF α -mediated disease or disorders.

[0080] In one aspect, the present invention provides a method for detecting the presence (or absence) or level of at least one isotype (*e.g.*, a plurality of isotypes) of an autoantibody to an anti-TNF α drug in a sample, the method comprising:

- (a) contacting a labeled anti-TNF α drug (*e.g.*, labeled anti-TNF α antibody)
20 with a sample having or suspected of having at least one isotype of an autoantibody to the anti-TNF α drug (*e.g.*, IgA, IgD, IgE, IgG, and/or IgM) to form labeled complexes (*i.e.*, immuno-complexes or conjugates) between the labeled anti-TNF α drug and each autoantibody isotype (*i.e.*, wherein the labeled anti-TNF α drug and autoantibody isotype are not covalently
25 attached to each other);
- (b) subjecting the labeled complexes to size exclusion chromatography to separate the labeled complexes having different autoantibody isotypes from each other and/or from free labeled anti-TNF α drug; and
- (c) detecting the labeled complexes, thereby detecting the presence (or
30 absence) or level of at least one isotype of the autoantibody to the anti-TNF α drug.

[0081] In one related aspect, the present invention provides a method for detecting the presence (or absence) or level of at least one isotype (*e.g.*, a plurality of isotypes) of an autoantibody to an anti-TNF α drug in a sample, the method comprising:

- 5 (a) contacting a labeled anti-TNF α drug (*e.g.*, labeled anti-TNF α antibody) and one or a plurality of labeled anti-Ig antibodies specific for different antibody isotypes (*e.g.*, IgA, IgD, IgE, IgG, and/or IgM isotypes) with a sample having or suspected of having at least one isotype of an autoantibody to the anti-TNF α drug to form labeled complexes (*i.e.*, immuno-complexes or conjugates) between the labeled anti-TNF α drug, the
10 labeled anti-Ig antibodies, and each autoantibody isotype (*i.e.*, wherein the components of the labeled complexes are not covalently attached to each other), wherein the labeled anti-TNF α drug and the labeled anti-Ig antibodies comprise different labels;
- 15 (b) subjecting the labeled complexes to size exclusion chromatography to separate the labeled complexes having different autoantibody isotypes from each other, from free labeled anti-TNF α drug, and/or from free labeled anti-Ig antibodies; and
- (c) detecting the labeled complexes, thereby detecting the presence (or
20 absence) or level of at least one isotype of the autoantibody to the anti-TNF α drug.

[0082] In some embodiments, the anti-TNF α drug is selected from the group consisting of REMICADETM (infliximab), HUMIRATM (adalimumab), ENBRELTM (etanercept), CIMZIA[®] (certolizumab pegol), and combinations thereof.

[0083] In certain embodiments, the methods are useful for measuring the (concentration)
25 levels of at least one, two, three, four, five, or more antibody isotypes in a sample such as a whole blood, serum, or plasma sample from a subject receiving anti-TNF α drug therapy. In some instances, the methods are useful for determining different anti-drug antibody (ADA) isotypes such as different isotypes of ATI (*i.e.*, antibody to IFX; “HACA”) in ADA-positive patients receiving anti-TNF α drug therapy such as infliximab (IFX). In
30 other instances, the methods are useful for determining different ADA isotypes such as different isotypes of ATA (*i.e.*, antibody to adalimumab; “HAHA”) in ADA-positive patients receiving anti-TNF α drug therapy such as adalimumab. Non-limiting examples of antibody isotypes include IgA, IgD, IgE, IgG, and IgM. In particular embodiments, the

methods of the invention aid or assist in associating different clinical outcomes in patients receiving anti-TNF α drug therapy based upon the presence or level of a specific ADA isotype or a particular combination of ADA isotypes.

[0084] An anti-TNF α drug or an anti-Ig antibody specific for an antibody isotype can be
5 labeled with a variety of detectable group(s). In particular embodiments, the anti-TNF α
drug and/or the anti-Ig isotype-specific antibody is labeled with a fluorophore or a
fluorescent dye. Non-limiting examples of fluorophores suitable for use as labels that can
be attached to the anti-TNF α drugs and anti-Ig isotype-specific antibodies described herein
include those listed in the Molecular Probes Catalogue, which is herein incorporated by
10 reference in its entirety for all purposes (*see*, R. Haugland, *The Handbook-A Guide to
Fluorescent Probes and Labeling Technologies*, 10th Edition, Molecular probes, Inc.
(2005)). Such exemplary fluorophores 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,
15 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, without limitation, Dansyl
Chloride (DNS-Cl), 5-(iodoacetamida)fluorescein (5-IAF), fluorescein 5-isothiocyanate
(FITC), tetramethylrhodamine 5- (and 6-)isothiocyanate (TRITC), 6-acryloyl-2-
20 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,
25 Parinaric acid, TMA-DPH, Fluorenyl fatty acid, fluorescein-phosphatidylethanolamine,
Texas Red-phosphatidylethanolamine, Pyrenyl-phosphatidylcholine, Fluorenyl-
phosphatidylcholine, Merocyanine 540, 1-(3-sulfonatopropyl)-4-[β -2[(di-n-butylamino)-6
naphthyl]vinyl]pyridinium betaine (Naphtyl Styryl), 3,3'-dipropylthiadicarbocyanine (diS-
C₃-(5)), 4-(p-dipentyl aminostyryl)-1-methylpyridinium (di-5-ASP), Cy-3 Iodo Acetamide,
30 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 derivatives thereof. In one non-limiting example, the plurality of anti-Ig isotype-specific antibodies is each labeled with Alexa-532 and the anti-TNF α drug is labeled with Alexa-488.

[0085] Typically, the fluorescent group is a fluorophore selected from the category of dyes comprising polymethines, phthalocyanines, cyanines, xanthenes, fluorenes, rhodamines, coumarins, fluoresceins, and BODIPY[™].

[0086] In one embodiment, the fluorescent group is 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, the near-infrared (NIR) fluorophore is preferably selected from 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, the near infrared group is IRDye[®] 800CW, IRDye[®] 800, IRDye[®] 700DX, IRDye[®] 700, or Dynamic DY676.

[0087] 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-TNF α drug or an anti-Ig isotype-specific antibody results in a stable covalent bond formed between a fluorophore and the anti-TNF α drug or the anti-Ig isotype-specific antibody.

[0088] In certain instances, following a fluorescent labeling reaction, it is often
5 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.

[0089] Reactive fluorescent dyes are available from many sources. They can be obtained with different reactive groups for attachment to various functional groups within
10 the target molecule. They are also available in labeling kits that contain all the components to carry out a labeling reaction. In one particular embodiment, Alexa Fluor[®] 647 C2 maleimide is used from Invitrogen (Cat. No. A-20347).

[0090] Specific immunological binding of an anti-TNF α drug to an autoantibody isotype or of an anti-TNF α drug and an anti-Ig antibody to an autoantibody isotype can be detected
15 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-TNF α drug or an anti-Ig antibody labeled with iodine-125 (¹²⁵I) can be used for determining the presence or concentration levels of one or a plurality of ADA isotypes in a sample. In other instances, a chemiluminescence assay using a chemiluminescent anti-TNF α drug or
20 anti-Ig antibody is suitable for sensitive, non-radioactive detection of ADA isotypes in a sample. In particular instances, an anti-TNF α drug and/or an anti-Ig antibody labeled with a fluorochrome is also suitable for determining the concentration levels of one or a plurality of ADA isotypes in a sample. Examples of fluorochromes include, without limitation, Alexa Fluor[®] dyes, DAPI, fluorescein, Hoechst 33258, R-phycoerythrin, B-
25 phycoerythrin, R-phycoerythrin, rhodamine, Texas red, and lissamine. Secondary antibodies linked to fluorochromes can be obtained commercially. As a non-limiting example, goat F(ab')₂ anti-human IgG-FITC is available from Tago Immunologicals (Burlingame, CA).

[0091] Indirect labels include various enzymes well-known in the art, such as
30 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-
5 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
10 Grove, PA.).

[0092] 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 ¹²⁵I; or a fluorometer to detect fluorescence in the presence of light of a certain wavelength. For detection of enzyme-
15 linked antibodies, a quantitative analysis of the amount of one or a plurality of ADA isotypes 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.

20 [0093] In particular embodiments, size exclusion chromatography (SEC) is used in the assays of the invention. 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
25 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
30 molecules below this molecular mass are so small that they elute as a single band.

[0094] In certain embodiments, 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. In preferred embodiments, 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 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
5 molecular hydrodynamic volume (*i.e.*, heavier moieties come off first).

[0095] In some instances, the (concentration) level of the ADA isotype present in a sample such as a serum sample can be compared to a standard curve and/or one or more controls. In certain instances, a labeled anti-TNF α drug can be incubated with known amounts of an anti-Ig isotype-specific antibody in a liquid phase reaction to create a
10 standard curve.

[0096] In certain instances, the autoantibody isotyping methods of the present invention are proximity-based such that they rely upon a signal that is generated by the proximity binding of both the labeled anti-TNF α drug and the labeled anti-Ig isotype-specific antibody to the autoantibody isotype. In particular embodiments, the signal generated by
15 the proximity assay is a fluorescent signal that can be detected by fluorescence resonance energy transfer (FRET). In other embodiments, the signal is detected by another proximity-based method as described herein or known to one of skill in the art.

[0097] In some instances, the plurality of labeled anti-Ig antibodies specific for different antibody isotypes each comprise the same label. In other instances, the plurality of labeled
20 anti-Ig antibodies specific for different antibody isotypes each comprise different labels. A skilled person in the art will appreciate that anti-Ig antibodies that bind to specific classes or subclasses of antibodies (*e.g.*, IgA, IgD, IgE, IgG, or IgM isotypes, or Ig subclasses thereof such as IgG1, IgG2, IgG3, and/or IgG4) are commercially available from vendors such as, for example, Miltenyi Biotec GmbH, Jackson ImmunoResearch Laboratories,
25 Inc., Santa Cruz Biotechnology, Inc., Abcam plc, and others. In certain instances, the anti-Ig antibody detects a specific Ig subclass (*e.g.*, IgG1, IgG2, IgG3, or IgG4). In certain other instances, the anti-Ig antibody detects all Ig subclasses (*e.g.*, IgG1, IgG2, IgG3, and IgG4). For example, anti-IgA antibodies (*e.g.*, from clone IS11-8E10, which detects both subclasses of human IgA), anti-IgG antibodies (*e.g.*, from clone IS11-3B2.2.3, which
30 detects all subclasses of the human IgG isotype), and anti-IgM antibodies (*e.g.*, from clone PJ2-22H3, which detects the IgM isotype) can be obtained from Miltenyi Biotec GmbH and labeled by attaching a detectable label such as a fluorophore thereto using methods described herein and known to one of skill in the art. In some instances, Molecular Probes

kits from Life Technologies such as the Alexa Fluor Protein Labeling Kits and the Alexa Fluor Monoclonal Labeling Kits, which include amine-reactive Alexa Fluor dyes (*e.g.*, Alexa Fluor succinimidyl (NHS) esters and/or Alexa Fluor tetrafluorophenyl (TFP) esters), can be used to selectively link Alexa Fluor dyes to accessible primary amine groups on
5 antibodies.

[0098] The term “proximity” as used herein includes reference to the spatial nearness or closeness of an anti-TNF α drug to an anti-Ig isotype-specific antibody when both are bound to the same autoantibody isotype (*i.e.*, an anti-drug antibody isotype such as IgA ATI). In particular embodiments, the binding of an anti-TNF α drug to an autoantibody
10 isotype at a distance near or close to the binding of an anti-Ig isotype-specific antibody to the same autoantibody isotype is sufficient to generate a detectable signal. In some embodiments, the term “proximity” includes those distances between the labeled antibodies, when bound to the same autoantibody isotype, that are sufficient to generate a detectable signal. In certain other embodiments, the term “proximity” includes those
15 distances between the detectable labels (*e.g.*, fluorescent labels) attached to the antibodies bound to the same autoantibody isotype that are sufficient to generate a detectable signal.

[0099] FRET describes an energy transfer mechanism between two fluorescent molecules. When a fluorescent donor is excited at its specific fluorescence excitation wavelength, this excited state is nonradiatively transferred to a second molecule, the
20 acceptor, by a long-range dipole-dipole coupling mechanism. The donor then returns to the electronic ground state. *See, e.g.*, Lakowicz, Principles of Fluorescence Spectroscopy, Plenum Publishing Corp., 2nd Ed. (1999). In the context of the invention, the anti-TNF α drug (*e.g.*, IFX) can be labeled with a donor comprising a first fluorescent dye and the anti-Ig isotype-specific antibody (*e.g.*, anti-IgA, anti-IgG) can be labeled with an acceptor
25 comprising a second fluorescent dye that has a different excitation and emission spectra from the first fluorescent dye. Non-limiting examples of fluorescent dyes suitable for use as detectable labels are described above and include fluorophores such as Alexa Fluor[®] dyes (*e.g.*, 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[®]
30 568, 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) and other fluorophores such as, for example, fluorescein, FITC,

rhodamine, Texas Red, TRITC, Cy3, Cy5, Cy5.5, Cy7, derivatives thereof, and combinations thereof.

[0100] In some embodiments, a sample such as a serum sample that is to be interrogated for the presence (or absence) or level of the autoantibody isotype (*i.e.*, the isotype of the autoantibody to the anti-TNF α drug) is incubated with both the labeled anti-TNF α drug and the labeled anti-Ig isotype-specific antibody. If the autoantibody isotype (*e.g.*, IgA ADA) that is detected by the particular anti-Ig isotype-specific antibody (*e.g.*, anti-IgA) is not present in the sample, the donor emission is detected upon donor excitation. On the other hand, if the autoantibody isotype (*e.g.*, IgA ADA) that is detected by the particular anti-Ig isotype-specific antibody (*e.g.*, anti-IgA) is present in the sample, the donor and acceptor fluorophores are brought into proximity (*e.g.*, from about 1 to about 300 nm or from about 1 to about 200 nm of each other, such as, for example, about 1, 5, 10, 25, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300 nm or any range thereof, or from about 1 to about 10 nm, *e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 nm or any range thereof) due to the interaction of both the labeled anti-TNF α drug and the particular labeled anti-Ig isotype-specific antibody with the autoantibody isotype (*e.g.*, IgA ATI). The intermolecular FRET from the donor fluorophore to the acceptor fluorophore results in the acceptor emission being predominantly observed. For example, excitation at 480 nm for Alexa-488 as the donor fluorophore on the anti-TNF α drug induces formation of singlet oxygen molecules that react with thioxene derivatives, generating chemiluminescence, which in turn excites the acceptor fluorophore Alexa-532 on the anti-Ig isotype-specific antibody to emit at 575 nm.

[0101] Example 29 describes an exemplary proximity-based isotyping assay of the present invention for determining the presence (or absence) or level of at least one, two, three, four, five, or more ATI isotypes such as, *e.g.*, IgA ATI, IgD ATI, IgE ATI, IgG ATI, and/or IgM ATI isotypes using FRET. As a non-limiting example, IFX labeled with a first fluorophore (“F1”) and an anti-IgA antibody labeled with a second fluorophore (“F2”) are incubated with a sample such as a serum sample containing one or more ATI isotypes such as IgA ATI. In some embodiments, F2 is excited by F1 only when both fluorophores are in close proximity, and the presence and/or level of a ternary complex of F1-IFX, F2-anti-IgA, and IgA ATI is indicative of the presence and/or level of the IgA ATI isotype that is present in the sample.

[0102] In other embodiments, the signal that is generated by the autoantibody isotyping proximity assay is a fluorescent signal that can be detected using an electrophoretic technique such as capillary electrophoresis (CE). CE analysis generally occurs inside a small-diameter quartz capillary in the presence of high (kilovolt-level) separating voltages with separation times of a few minutes. In the context of the present invention, the anti-TNF α drug (*e.g.*, IFX) can be labeled with a photoactivatable enzymatic scissor and the anti-Ig isotype-specific antibody (*e.g.*, anti-IgA, anti-IgD, anti-IgE, anti-IgG, and/or anti-IgM) can be labeled with an electrophoretic tag reporter. Non-limiting examples of photoactivatable enzymatic scissors and electrophoretic tag reporters such as the *eTag*TM family of small fluorescent reporter molecules are described in U.S. Patent No. 6,673,550. The binding of the labeled anti-TNF α drug to the autoantibody isotype (*e.g.*, IgA ATI) in proximity (*e.g.*, from about 1 to about 300 nm or from about 1 to about 200 nm of each other, such as, for example, about 1, 5, 10, 25, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300 nm or any range thereof, or from about 1 to about 10 nm, *e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 nm or any range thereof) of the binding of the labeled anti-Ig isotype-specific antibody to the same autoantibody isotype triggers the cleavage of the electrophoretic tag reporter by the photoactivatable enzymatic scissor. Following photoactive cleavage, released electrophoretic tag reporter molecules are analyzed by CE separation as described in, *e.g.*, Chan-Hui *et al.*, *Clin. Immun.*, 111:162-174 (2004).

Alternatively, the anti-TNF α drug can be labeled with an electrophoretic tag reporter and the anti-Ig isotype-specific antibody can be labeled with a photoactivatable enzymatic scissor. In some embodiments, a plurality of electrophoretic tag reporters can be attached to a plurality of distinct anti-Ig isotype-specific antibodies and used in a multiplex assay to determine the presence (or absence) or level of multiple autoantibody isotypes of interest.

[0103] In yet other embodiments, the signal that is generated by the autoantibody isotyping proximity assay is a DNA amplification signal that can be detected using a nucleic acid amplification technique such as the polymerase chain reaction (PCR). The use of PCR for the amplification of nucleic acids is well known in the art and is described in, *e.g.*, Mullis *et al.*, *The Polymerase Chain Reaction*, Birkhäuser, Boston (1994); and Innis *et al.*, *PCR Applications: Protocols for Functional Genomics*, 1st Ed., Academic Press (1999). In the context of the present invention, the anti-TNF α drug (*e.g.*, IFX) can be labeled with a first oligonucleotide extension and the anti-Ig isotype-specific antibody (*e.g.*, anti-IgA, anti-IgD, anti-IgE, anti-IgG, and/or anti-IgM) can be labeled with a second

oligonucleotide extension. If the autoantibody isotype (*e.g.*, IgA ATI) that is detected by the particular anti-Ig isotype-specific antibody (*e.g.*, anti-IgA) is not present in the sample, a connector oligonucleotide independently hybridizes to each oligonucleotide extension, which does not promote their ligation. On the other hand, if the autoantibody isotype (*e.g.*, IgA ATI) that is detected by the particular anti-Ig isotype-specific antibody (*e.g.*, anti-IgA) is present in the sample, the binding of the labeled anti-TNF α drug to the autoantibody isotype (*e.g.*, IgA ATI) in proximity (*e.g.*, from about 1 to about 300 nm or from about 1 to about 200 nm of each other, such as, for example, about 1, 5, 10, 25, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300 nm or any range thereof, or from about 1 to about 10 nm, *e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 nm or any range thereof) of the binding of the labeled anti-Ig isotype-specific antibody to the same autoantibody isotype enables the connector oligonucleotide to simultaneously hybridize to both oligonucleotide extensions, thereby triggering the ligation of the oligonucleotide extensions by DNA ligase. One of skill in the art will appreciate that the proximity of the anti-TNF α drug to the anti-Ig isotype-specific antibody may vary depending on the length of the oligonucleotide extension. Thus, a new species of DNA sequence is created, not previously present in the reaction, which can be amplified using standard DNA amplification techniques such as PCR. The oligonucleotide extensions and connector oligonucleotides are typically chemically synthesized by any method known in the art and independently comprise a nucleotide sequence of at least about 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, or 60 nucleotides in length.

[0104] In further embodiments, the signal that is generated by the autoantibody isotyping proximity assay is a chemiluminescent or fluorescent signal that can be detected using signal amplification such as tyramide signal amplification. In the context of the present invention, the anti-TNF α drug (*e.g.*, IFX) can be labeled with glucose oxidase (GO) or any other enzyme that catalyzes an oxidation/reduction reaction involving molecular oxygen (O₂) as the electron acceptor. The anti-Ig isotype-specific antibody (*e.g.*, anti-IgA, anti-IgD, anti-IgE, anti-IgG, and/or anti-IgM) can be labeled with a peroxidase such as horseradish peroxidase (HRP), *e.g.*, directly or indirectly via binding pair members such as biotin/streptavidin. Other examples of peroxidases include, but are not limited to, catalase, chloroperoxidase, cytochrome c peroxidase, eosinophil peroxidase, glutathione peroxidase, lactoperoxidase, myeloperoxidase, thyroid peroxidase, deiodinase, and the like. When the GO is contacted with a substrate such as glucose, it generates an oxidizing agent (*i.e.*,

hydrogen peroxide (H₂O₂)). If the autoantibody isotype (*e.g.*, IgA ATI) that is detected by the particular anti-Ig isotype-specific antibody (*e.g.*, anti-IgA) is present in the sample, the binding of the labeled anti-TNF α drug to the autoantibody isotype (*e.g.*, IgA ATI) in proximity (*e.g.*, from about 1 to about 300 nm or from about 1 to about 200 nm of each other, such as, for example, about 1, 5, 10, 25, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300 nm or any range thereof, or from about 1 to about 10 nm, *e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 nm or any range thereof) of the binding of the labeled anti-Ig isotype-specific antibody to the same autoantibody isotype enables the H₂O₂ generated by the GO to channel to and complex with the HRP to form an HRP-H₂O₂ complex, which, in the presence of a chemiluminescent substrate (*e.g.*, luminol, isoluminol) or a fluorogenic substrate (*e.g.*, tyramide, biotin-tyramide, homovanillic acid, 4-hydroxyphenyl acetic acid), generates an amplified signal.

[0105] Methods of using GO and HRP in a proximity assay are described in, *e.g.*, PCT Patent Publication No. WO 2008/036802, the disclosure of which is hereby incorporated by reference in its entirety for all purposes. When biotin-tyramide is used as the fluorogenic substrate, the HRP-H₂O₂ complex oxidizes the tyramide to generate a reactive tyramide radical that covalently binds nearby nucleophilic residues. The activated tyramide is either directly detected or detected upon the addition of a signal-detecting reagent such as, for example, a streptavidin-labeled fluorophore or a combination of a streptavidin-labeled peroxidase and a chromogenic reagent. Examples of fluorophores suitable for use in the present invention include, but are not limited to, any of the fluorophores described herein including an Alexa Fluor[®] dye (*e.g.*, 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), fluorescein, fluorescein isothiocyanate (FITC), Oregon Green[™], rhodamine, Texas red, tetra-rhodamine isothiocyanate (TRITC), a CyDye[™] fluor (*e.g.*, Cy2, Cy3, Cy5), and the like. The streptavidin label can be coupled directly or indirectly to the fluorophore or peroxidase using methods well-known in the art. Non-limiting examples of chromogenic reagents include 3,3',5,5'-tetramethylbenzidine (TMB), 3,3'-diaminobenzidine (DAB), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 4-chloro-1-naphthol (4CN), and/or porphyrinogen.

[0106] Additional proximity-based techniques suitable for use in the assay methods of the present invention for autoantibody isotyping are described in PCT Patent Publication No. WO 2008/036802, the disclosure of which is hereby incorporated by reference in its entirety for all purposes.

5 **[0107]** Methods for detecting anti-TNF antibodies and anti-drug antibodies such as HACA and HAHA 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.

[0108] In another 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

10 TNF α -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); and

15 (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 statistical algorithm applied in step (b).

[0109] In some embodiments, the course of therapy comprises an anti-TNF α drug such as an anti-TNF α antibody. In certain instances, the anti-TNF α antibody is selected from

20 the group consisting of REMICADE™ (infliximab), ENBREL™ (etanercept), HUMIRA™ (adalimumab), CIMZIA® (certolizumab pegol), and combinations thereof.

[0110] In certain embodiments, the one or more (*e.g.*, a plurality of) markers comprise an anti-TNF α drug (*e.g.*, anti-TNF α antibody), an autoantibody to an anti-TNF α drug such as an anti-TNF α antibody (*e.g.*, HACA, HAHA, HAMA, and/or isotypes thereof), a

25 cytokine, a genetic marker, or combinations thereof. In certain instances, the cytokine is a member selected from the group consisting of TNF α , IL-6, IL-1 β , IFN- γ , IL-10, and combinations thereof. In certain other instances, the genetic marker is a mutation in an inflammatory pathway gene. In particular embodiments, the genetic marker is a mutation in a gene selected from the group consisting of NOD2/CARD15, ATG16L1, IL23R, a

30 human leukocyte antigen (HLA) gene, a cytokine gene, DLG5, OCTN, and combinations thereof.

[0111] In other embodiments, step (a) of the method for optimizing therapy and/or reducing toxicity comprises determining the presence, level, or genotype of two, three, four, five, six, seven, eight, nine, ten, or more markers in said sample. In certain embodiments, the sample is selected from the group consisting of serum, plasma, whole
5 blood, and stool.

[0112] In yet other embodiments, the statistical algorithm comprises a learning statistical classifier system. In certain 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
10 detector model, interactive tree, multiadaptive regression spline, machine learning classifier, and combinations thereof. In some embodiments, the statistical algorithm comprises a single learning statistical classifier system. In other embodiments, the statistical algorithm comprises a combination of at least two learning statistical classifier systems. In some instances, at least two learning statistical classifier systems are applied
15 in tandem.

[0113] In some embodiments, the method for optimizing therapy and/or reducing toxicity further comprises sending the results from said determination of step (c) to a clinician. In other embodiments, step (b) of the method for optimizing therapy and/or reducing toxicity further comprises applying the statistical algorithm to the presence, level,
20 or genotype of the one or more markers determined at an earlier time during the course of therapy.

[0114] In further embodiments, the subsequent dose of the course of therapy is increased, decreased, or maintained based upon the statistical algorithm applied in step (b). In certain instances, the different course of therapy comprises a different anti-TNF α drug
25 (*e.g.*, anti-TNF α antibody). In other instances, the different course of therapy comprises the current course of therapy along with an immunosuppressive agent.

[0115] In some embodiments, the anti-TNF α drug (*e.g.*, anti-TNF α antibody) is detected with an assay comprising:

- (a) contacting labeled TNF α with the sample to form a labeled complex
30 with the anti-TNF α drug;
- (b) subjecting the labeled complex to size exclusion chromatography to separate the labeled complex; and

- (c) detecting the labeled complex, thereby detecting the anti-TNF α drug.
- [0116]** In certain instances, the anti-TNF α drug (*e.g.*, anti-TNF α antibody) is a member selected from the group consisting of REMICADE™ (infliximab), ENBREL™ (etanercept), HUMIRA™ (adalimumab), CIMZIA® (certolizumab pegol), and combinations thereof. In some instances, the labeled TNF α is a fluorophore labeled TNF α . In other instances, the detected anti-TNF α drug (*e.g.*, anti-TNF α antibody) is quantitated. In some embodiments, the labeled complex is eluted first, followed by free labeled TNF α . In other embodiments, the size exclusion chromatography is size exclusion-high performance liquid chromatography (SE-HPLC).
- [0117]** In other embodiments, the autoantibody to the anti-TNF α drug (*e.g.*, anti-TNF α antibody) is detected with an assay comprising:
- (a) contacting labeled anti-TNF α drug with the sample to form a labeled complex with the autoantibody;
 - (b) subjecting the labeled complex to size exclusion chromatography to separate the labeled complex; and
 - (c) detecting the labeled complex, thereby detecting the autoantibody.
- [0118]** In certain instances, the autoantibody is selected from the group consisting of human anti-mouse antibody (HAMA), human anti-chimeric antibody (HACA), human anti-humanized antibody (HAHA), and combinations thereof. In certain other instances, the labeled anti-TNF α drug (*e.g.*, anti-TNF α antibody) is selected from the group consisting of REMICADE™ (infliximab), ENBREL™ (etanercept), HUMIRA™ (adalimumab), CIMZIA® (certolizumab pegol), and combinations thereof.
- [0119]** In some embodiments, the labeled anti-TNF α drug (*e.g.*, anti-TNF α antibody) is a fluorophore labeled anti-TNF α drug (*e.g.*, anti-TNF α antibody). In other embodiments, the detected autoantibody is quantitated. In further embodiments, the labeled complex is eluted first, followed by free labeled anti-TNF α drug (*e.g.*, anti-TNF α antibody). In particular embodiments, the size exclusion chromatography is size exclusion-high performance liquid chromatography (SE-HPLC).
- [0120]** In certain embodiments, the autoantibody to the anti-TNF α drug (*e.g.*, anti-TNF α antibody) is detected with any of the assays described herein for detecting the presence (or

absence) or level of at least one isotype (*e.g.*, a plurality of isotypes) of an autoantibody to an anti-TNF α drug.

[0121] In other embodiments, the assay methods described in PCT Publication No. WO 2011/056590 (the disclosure of which is hereby incorporated by reference in its entirety for
5 all purposes) for detecting anti-TNF α drugs (*e.g.*, anti-TNF α antibodies) and autoantibodies to anti-TNF α drugs (*e.g.*, HACA, HAHA, HAMA, *etc.*) can be used in the methods described herein for optimizing therapy and/or reducing toxicity.

[0122] In certain embodiments, the methods of the present invention provide information useful for guiding treatment decisions for patients receiving anti-TNF drug
10 therapy, *e.g.*, by determining when or how to adjust or modify (*e.g.*, increase or decrease) the subsequent dose of an anti-TNF drug, by determining when or how to combine an anti-TNF drug (*e.g.*, at an increased, decreased, or same dose) with one or more immunosuppressive agents such as methotrexate (MTX) or azathioprine (AZA), and/or by
15 determining when or how to change the current course of therapy (*e.g.*, switch to a different anti-TNF drug). Accordingly, the present invention finds utility in aiding or assisting in patient management by determining patient immune status.

[0123] In other embodiments, the assay methods described herein can be used to predict tolerability (*e.g.*, toxicity) to an anti-TNF drug, especially to an anti-TNF α antibody in a subject having an autoimmune disorder (*e.g.*, rheumatoid arthritis, Crohn's Disease, and
20 the like). In this method, by assaying the subject for the presence (or absence) or level of one or more ADA isotypes, it is possible to predict whether the subject will tolerate the anti-TNF drug therapy (*e.g.*, not develop or experience side-effects such as an immune response to the anti-TNF drug).

[0124] In yet other embodiments, the assay methods described herein can be used to
25 monitor an autoimmune disorder in a subject having the autoimmune disorder comprising assaying the subject for the presence (or absence) or level of one or more ADA isotypes over time. In this method, it is possible to monitor whether the subject tolerates the anti-TNF drug therapy over the given time period.

IV. Statistical Analysis

30 [0125] In some aspects, the present invention provides methods for optimizing anti-TNF drug therapy, reducing toxicity associated with anti-TNF drug therapy, and/or monitoring

the efficacy of anti-TNF drug treatment by applying a statistical algorithm to one or more (e.g., a combination of two, three, four, five, six, seven, or more) biochemical markers, serological markers, and/or genetic markers. In particular embodiments, quantile analysis is applied to the presence, level, and/or genotype of one or more markers to guide treatment decisions for patients receiving anti-TNF drug therapy. In other embodiments, one or a combination of two or more learning statistical classifier systems are applied to the presence, level, and/or genotype of one or more markers to guide treatment decisions for patients receiving anti-TNF drug therapy. The statistical analyses of the methods of the present invention advantageously provide improved sensitivity, specificity, negative predictive value, positive predictive value, and/or overall accuracy for determining when or how to adjust or modify (e.g., increase or decrease) the subsequent dose of an anti-TNF drug, to combine an anti-TNF drug (e.g., at an increased, decreased, or same dose) with one or more immunosuppressive agents such as methotrexate (MTX) or azathioprine (AZA), and/or to change the current course of therapy (e.g., switch to a different anti-TNF drug).

[0126] The term “statistical analysis” or “statistical algorithm” or “statistical process” includes any of a variety of statistical methods and models used to determine relationships between variables. In the present invention, the variables are the presence, level, or genotype of at least one marker of interest. Any number of markers can be analyzed using a statistical analysis described herein. For example, the presence or level of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, or more markers can be included in a statistical analysis. In one embodiment, logistic regression is used. In another embodiment, linear regression is used. In certain preferred embodiments, the statistical analyses of the present invention comprise a quantile measurement of one or more markers, e.g., within a given population, as a variable. Quantiles are a set of “cut points” that divide a sample of data into groups containing (as far as possible) equal numbers of observations. For example, quartiles are values that divide a sample of data into four groups containing (as far as possible) equal numbers of observations. The lower quartile is the data value a quarter way up through the ordered data set; the upper quartile is the data value a quarter way down through the ordered data set. Quintiles are values that divide a sample of data into five groups containing (as far as possible) equal numbers of observations. The present invention can also include the use of percentile ranges of marker levels (e.g., tertiles, quartile, quintiles, *etc.*), or their cumulative indices (e.g.,

quartile sums of marker levels to obtain quartile sum scores (QSS), *etc.*) as variables in the statistical analyses (just as with continuous variables).

[0127] In certain embodiments, the present invention involves detecting or determining the presence, level (*e.g.*, magnitude), and/or genotype of one or more markers of interest using quartile analysis. In this type of statistical analysis, the level of a marker of interest is defined as being in the first quartile (<25%), second quartile (25-50%), third quartile (51%-<75%), or fourth quartile (75-100%) in relation to a reference database of samples. These quartiles may be assigned a quartile score of 1, 2, 3, and 4, respectively. In certain instances, a marker that is not detected in a sample is assigned a quartile score of 0 or 1, while a marker that is detected (*e.g.*, present) in a sample (*e.g.*, sample is positive for the marker) is assigned a quartile score of 4. In some embodiments, quartile 1 represents samples with the lowest marker levels, while quartile 4 represent samples with the highest marker levels. In other embodiments, quartile 1 represents samples with a particular marker genotype (*e.g.*, wild-type allele), while quartile 4 represent samples with another particular marker genotype (*e.g.*, allelic variant). The reference database of samples can include a large spectrum of patients with a TNF α -mediated disease or disorder such as, *e.g.*, IBD. From such a database, quartile cut-offs can be established. A non-limiting example of quartile analysis suitable for use in the present invention is described in, *e.g.*, Mow *et al.*, *Gastroenterology*, 126:414-24 (2004).

[0128] In some embodiments, the statistical analyses of the present invention comprise one or more learning statistical classifier systems. As used herein, the term “learning statistical classifier system” includes a machine learning algorithmic technique capable of adapting to complex data sets (*e.g.*, panel of markers of interest) and making decisions based upon such data sets. In some embodiments, a single learning statistical classifier system such as a decision/classification tree (*e.g.*, random forest (RF) or classification and regression tree (C&RT)) is used. In other embodiments, a combination of 2, 3, 4, 5, 6, 7, 8, 9, 10, or more learning statistical classifier systems are used, preferably in tandem. Examples of learning statistical classifier systems include, but are not limited to, those using inductive learning (*e.g.*, decision/classification trees such as random forests, classification and regression trees (C&RT), boosted trees, *etc.*), Probably Approximately Correct (PAC) learning, connectionist learning (*e.g.*, neural networks (NN), artificial neural networks (ANN), neuro fuzzy networks (NFN), network structures, perceptrons such as multi-layer perceptrons, multi-layer feed-forward networks, applications of neural

networks, Bayesian learning in belief networks, *etc.*), reinforcement learning (*e.g.*, passive learning in a known environment such as naïve learning, adaptive dynamic learning, and temporal difference learning, passive learning in an unknown environment, active learning in an unknown environment, learning action-value functions, applications of reinforcement learning, *etc.*), and genetic algorithms and evolutionary programming. Other learning statistical classifier systems include support vector machines (*e.g.*, Kernel methods), multivariate adaptive regression splines (MARS), Levenberg-Marquardt algorithms, Gauss-Newton algorithms, mixtures of Gaussians, gradient descent algorithms, and learning vector quantization (LVQ).

10 **[0129]** Random forests are learning statistical classifier systems that are constructed using an algorithm developed by Leo Breiman and Adele Cutler. Random forests use a large number of individual decision trees and decide the class by choosing the mode (*i.e.*, most frequently occurring) of the classes as determined by the individual trees. Random forest analysis can be performed, *e.g.*, using the RandomForests software available from
15 Salford Systems (San Diego, CA). *See, e.g.*, Breiman, *Machine Learning*, 45:5-32 (2001); and http://stat-www.berkeley.edu/users/breiman/RandomForests/cc_home.htm, for a description of random forests.

[0130] Classification and regression trees represent a computer intensive alternative to fitting classical regression models and are typically used to determine the best possible
20 model for a categorical or continuous response of interest based upon one or more predictors. Classification and regression tree analysis can be performed, *e.g.*, using the C&RT software available from Salford Systems or the Statistica data analysis software available from StatSoft, Inc. (Tulsa, OK). A description of classification and regression trees is found, *e.g.*, in Breiman *et al.* "Classification and Regression Trees," Chapman and
25 Hall, New York (1984); and Steinberg *et al.*, "CART: Tree-Structured Non-Parametric Data Analysis," Salford Systems, San Diego, (1995).

[0131] Neural networks are interconnected groups of artificial neurons that use a mathematical or computational model for information processing based on a connectionist approach to computation. Typically, neural networks are adaptive systems that change
30 their structure based on external or internal information that flows through the network. Specific examples of neural networks include feed-forward neural networks such as perceptrons, single-layer perceptrons, multi-layer perceptrons, backpropagation networks, ADALINE networks, MADALINE networks, Learnmatrix networks, radial basis function

(RBF) networks, and self-organizing maps or Kohonen self-organizing networks; recurrent neural networks such as simple recurrent networks and Hopfield networks; stochastic neural networks such as Boltzmann machines; modular neural networks such as committee of machines and associative neural networks; and other types of networks such as

5 instantaneously trained neural networks, spiking neural networks, dynamic neural networks, and cascading neural networks. Neural network analysis can be performed, *e.g.*, using the Statistica data analysis software available from StatSoft, Inc. *See, e.g.*, Freeman *et al.*, In “Neural Networks: Algorithms, Applications and Programming Techniques,” Addison-Wesley Publishing Company (1991); Zadeh, *Information and Control*, 8:338-353

10 (1965); Zadeh, “IEEE Trans. on Systems, Man and Cybernetics,” 3:28-44 (1973); Gersho *et al.*, In “Vector Quantization and Signal Compression,” Kluwer Academic Publishers, Boston, Dordrecht, London (1992); and Hassoun, “Fundamentals of Artificial Neural Networks,” MIT Press, Cambridge, Massachusetts, London (1995), for a description of neural networks.

15 **[0132]** Support vector machines are a set of related supervised learning techniques used for classification and regression and are described, *e.g.*, in Cristianini *et al.*, “An Introduction to Support Vector Machines and Other Kernel-Based Learning Methods,” Cambridge University Press (2000). Support vector machine analysis can be performed, *e.g.*, using the SVM^{light} software developed by Thorsten Joachims (Cornell University) or

20 using the LIBSVM software developed by Chih-Chung Chang and Chih-Jen Lin (National Taiwan University).

[0133] The various statistical methods and models described herein can be trained and tested using a cohort of samples (*e.g.*, serological and/or genomic samples) from healthy individuals and patients with a TNF α -mediated disease or disorder such as, *e.g.*, IBD (*e.g.*,

25 CD and/or UC). For example, samples from patients diagnosed by a physician, preferably by a gastroenterologist, as having IBD or a clinical subtype thereof using a biopsy, colonoscopy, or an immunoassay as described in, *e.g.*, U.S. Patent No. 6,218,129, are suitable for use in training and testing the statistical methods and models of the present invention. Samples from patients diagnosed with IBD can also be stratified into Crohn’s

30 disease or ulcerative colitis using an immunoassay as described in, *e.g.*, U.S. Patent Nos. 5,750,355 and 5,830,675. Samples from healthy individuals can include those that were not identified as IBD samples. One skilled in the art will know of additional techniques

and diagnostic criteria for obtaining a cohort of patient samples that can be used in training and testing the statistical methods and models of the present invention.

[0134] As used herein, the term “sensitivity” includes the probability that a method of the present invention for optimizing anti-TNF drug therapy, reducing toxicity associated with anti-TNF drug therapy, and/or monitoring the efficacy of anti-TNF drug treatment gives a positive result when the sample is positive, *e.g.*, having the predicted therapeutic response to anti-TNF drug therapy or toxicity associated with anti-TNF drug therapy. Sensitivity is calculated as the number of true positive results divided by the sum of the true positives and false negatives. Sensitivity essentially is a measure of how well the present invention correctly identifies those who have the predicted therapeutic response to anti-TNF drug therapy or toxicity associated with anti-TNF drug therapy from those who do not have the predicted therapeutic response or toxicity. The statistical methods and models can be selected such that the sensitivity is at least about 60%, and can be, *e.g.*, at least about 65%, 70%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%.

[0135] The term “specificity” includes the probability that a method of the present invention for optimizing anti-TNF drug therapy, reducing toxicity associated with anti-TNF drug therapy, and/or monitoring the efficacy of anti-TNF drug treatment gives a negative result when the sample is not positive, *e.g.*, not having the predicted therapeutic response to anti-TNF drug therapy or toxicity associated with anti-TNF drug therapy. Specificity is calculated as the number of true negative results divided by the sum of the true negatives and false positives. Specificity essentially is a measure of how well the present invention excludes those who do not have the predicted therapeutic response to anti-TNF drug therapy or toxicity associated with anti-TNF drug therapy from those who do have the predicted therapeutic response or toxicity. The statistical methods and models can be selected such that the specificity is at least about 60%, and can be, *e.g.*, at least about 65%, 70%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%.

[0136] The term “negative predictive value” or “NPV” includes the probability that an individual identified as not having the predicted therapeutic response to anti-TNF drug therapy or toxicity associated with anti-TNF drug therapy actually does not have the predicted therapeutic response or toxicity. Negative predictive value can be calculated as the number of true negatives divided by the sum of the true negatives and false negatives.

Negative predictive value is determined by the characteristics of the methods of the present invention as well as the prevalence of the disease in the population analyzed. The statistical methods and models can be selected such that the negative predictive value in a population having a disease prevalence is in the range of about 70% to about 99% and can be, for example, at least about 70%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%.

[0137] The term “positive predictive value” or “PPV” includes the probability that an individual identified as having the predicted therapeutic response to anti-TNF drug therapy or toxicity associated with anti-TNF drug therapy actually has the predicted therapeutic response or toxicity. Positive predictive value can be calculated as the number of true positives divided by the sum of the true positives and false positives. Positive predictive value is determined by the characteristics of the methods of the present invention as well as the prevalence of the disease in the population analyzed. The statistical methods and models can be selected such that the positive predictive value in a population having a disease prevalence is in the range of about 70% to about 99% and can be, for example, at least about 70%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%.

[0138] Predictive values, including negative and positive predictive values, are influenced by the prevalence of the disease in the population analyzed. In the present invention, the statistical methods and models can be selected to produce a desired clinical parameter for a clinical population with a particular prevalence for a TNF α -mediated disease or disorder such as, *e.g.*, IBD. As a non-limiting example, statistical methods and models can be selected for an IBD prevalence of up to about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, or 70%, which can be seen, *e.g.*, in a clinician’s office such as a gastroenterologist’s office or a general practitioner’s office.

[0139] As used herein, the term “overall agreement” or “overall accuracy” includes the accuracy with which a method of the present invention optimizes anti-TNF drug therapy, reduces toxicity associated with anti-TNF drug therapy, and/or monitors the efficacy of anti-TNF drug treatment. Overall accuracy is calculated as the sum of the true positives and true negatives divided by the total number of sample results and is affected by the prevalence of the disease in the population analyzed. For example, the statistical methods

and models can be selected such that the overall accuracy in a patient population having a disease prevalence is at least about 40%, and can be, *e.g.*, at least about 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%.

V. Inflammatory Markers

[0140] A variety of inflammatory markers, 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 optimizing therapy, reducing toxicity, and/or monitoring the efficacy of therapeutic treatment with therapeutic agents such as biologics (*e.g.*, anti-TNF 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 of the inflammatory markers to aid or assist in optimizing anti-TNF drug therapy, reducing toxicity associated with anti-TNF drug therapy, or monitoring the efficacy of therapeutic treatment with an anti-TNF drug.

[0141] Non-limiting examples of inflammatory markers include: (i) biochemical, serological, and protein markers such as, *e.g.*, cytokines, acute phase proteins, cellular adhesion molecules, and combinations thereof; and (ii) genetic markers such as, *e.g.*, any of the genes set forth in Table 1 (*e.g.*, NOD2).

A. Cytokines

[0142] The determination of the presence or level of at least one cytokine in a sample is particularly useful in the present 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, hematopoiesis, angiogenesis, wound healing, insulin resistance, the immune response, and the inflammatory response.

[0143] In certain aspects, the presence or level of at least one cytokine including, but not limited to, TNF- α , TNF-related weak inducer of apoptosis (TWEAK), osteoprotegerin

(OPG), IFN- α , IFN- β , IFN- γ , IL-1 α , IL-1 β , IL-1 receptor antagonist (IL-1ra), IL-2, IL-4, IL-5, IL-6, soluble IL-6 receptor (sIL-6R), IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, IL-23, and IL-27 is determined in a sample. In certain other aspects, the presence or level of at least one chemokine such as, for example, CXCL1/GRO1/GRO α ,
5 CXCL2/GRO2, CXCL3/GRO3, CXCL4/PF-4, CXCL5/ENA-78, CXCL6/GCP-2, CXCL7/NAP-2, CXCL9/MIG, CXCL10/IP-10, CXCL11/I-TAC, CXCL12/SDF-1, CXCL13/BCA-1, CXCL14/BRAK, CXCL15, CXCL16, CXCL17/DMC, CCL1, CCL2/MCP-1, CCL3/MIP-1 α , CCL4/MIP-1 β , CCL5/RANTES, CCL6/C10, CCL7/MCP-3, CCL8/MCP-2, CCL9/CCL10, CCL11/Eotaxin, CCL12/MCP-5, CCL13/MCP-4,
10 CCL14/HCC-1, CCL15/MIP-5, CCL16/LEC, CCL17/TARC, CCL18/MIP-4, CCL19/MIP-3 β , CCL20/MIP-3 α , CCL21/SLC, CCL22/MDC, CCL23/MPIF1, CCL24/Eotaxin-2, CCL25/TECK, CCL26/Eotaxin-3, CCL27/CTACK, CCL28/MEC, CL1, CL2, and CX₃CL1 is determined in a sample. In certain further aspects, the presence or level of at least one adipocytokine including, but not limited to, leptin, adiponectin,
15 resistin, active or total plasminogen activator inhibitor-1 (PAI-1), visfatin, and retinol binding protein 4 (RBP4) is determined in a sample. Preferably, the presence or level of TNF α , IL-6, IL-1 β , IFN- γ , and/or IL-10 is determined.

[0144] In certain instances, the presence or level of a particular cytokine is detected at the level of mRNA expression with an assay such as, for example, a hybridization assay or
20 an amplification-based assay. In certain other instances, the presence or level of a particular cytokine 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 cytokine such as IL-6, IL-1 β , or TWEAK in a serum, plasma, saliva, or urine sample are available from, *e.g.*, R&D Systems, Inc.
25 (Minneapolis, MN), Neogen Corp. (Lexington, KY), Alpco Diagnostics (Salem, NH), Assay Designs, Inc. (Ann Arbor, MI), BD Biosciences Pharmingen (San Diego, CA), Invitrogen (Camarillo, CA), Calbiochem (San Diego, CA), CHEMICON International, Inc. (Temecula, CA), Antigenix America Inc. (Huntington Station, NY), QIAGEN Inc. (Valencia, CA), Bio-Rad Laboratories, Inc. (Hercules, CA), and/or Bender MedSystems
30 Inc. (Burlingame, CA).

[0145] 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.

[0146] The human IL-1 β polypeptide sequence is set forth in, *e.g.*, Genbank Accession No. NP_000567. The human IL-1 β mRNA (coding) sequence is set forth in, *e.g.*,

5 Genbank Accession No. NM_000576. One skilled in the art will appreciate that IL-1 β is also known as IL1F2 and IL-1beta.

[0147] 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

10 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.

B. Acute Phase Proteins

[0148] The determination of the presence or level of one or more acute-phase proteins in
15 a sample is also useful in the present 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,

20 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
25 albumin, transferrin, transthyretin, transcortin, retinol-binding protein, and combinations thereof. Preferably, the presence or level of CRP and/or SAA is determined.

[0149] In certain instances, the presence or level of a particular acute-phase 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

30 presence or level of a particular acute-phase protein is detected at the level of protein expression using, for example, an immunoassay (*e.g.*, ELISA) or an immunohistochemical assay. For example, a sandwich colorimetric ELISA assay available from Alpco

Diagnostics (Salem, NH) can be used to determine the level of CRP in a serum, plasma, urine, or stool sample. Similarly, an ELISA kit available from Biomeda Corporation (Foster City, CA) can be used to detect CRP levels in a sample. Other methods for determining CRP levels in a sample are described in, *e.g.*, U.S. Patent Nos. 6,838,250 and 6,406,862; and U.S. Patent Publication Nos. 20060024682 and 20060019410. Additional methods for determining CRP levels include, *e.g.*, immunoturbidimetry assays, rapid immunodiffusion assays, and visual agglutination assays. Suitable ELISA kits for determining the presence or level of SAA in a sample such as serum, plasma, saliva, urine, or stool are available from, *e.g.*, Antigenix America Inc. (Huntington Station, NY), Abazyme (Needham, MA), USCN Life (Missouri City, TX), and/or U.S. Biological (Swampscott, MA).

[0150] C-reactive protein (CRP) is a protein found in the blood in response to inflammation (an acute-phase protein). CRP is typically produced by the liver and by fat cells (adipocytes). It is a member of the pentraxin family of proteins. The human CRP polypeptide sequence is set forth in, *e.g.*, Genbank Accession No. NP_000558. The human CRP mRNA (coding) sequence is set forth in, *e.g.*, Genbank Accession No. NM_000567. One skilled in the art will appreciate that CRP is also known as PTX1, MGC88244, and MGC149895.

[0151] Serum amyloid A (SAA) proteins are a family of apolipoproteins associated with high-density lipoprotein (HDL) in plasma. Different isoforms of SAA are expressed constitutively (constitutive SAAs) at different levels or in response to inflammatory stimuli (acute phase SAAs). These proteins are predominantly produced by the liver. The conservation of these proteins throughout invertebrates and vertebrates suggests SAAs play a highly essential role in all animals. Acute phase serum amyloid A proteins (A-SAAs) are secreted during the acute phase of inflammation. The human SAA polypeptide sequence is set forth in, *e.g.*, Genbank Accession No. NP_000322. The human SAA mRNA (coding) sequence is set forth in, *e.g.*, Genbank Accession No. NM_000331. One skilled in the art will appreciate that SAA is also known as PIG4, TP53I4, MGC111216, and SAA1.

30 **C. Cellular Adhesion Molecules (IgSF CAMs)**

[0152] 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 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.

1. Intercellular Adhesion Molecule-1 (ICAM-1)

[0153] 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 TNF α 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).

2. Vascular Cell Adhesion Molecule-1 (VCAM-1)

[0154] 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 (TNF α) 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)).

[0155] 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).

15 **D. Genetic Markers**

[0156] The determination of the presence or absence of allelic variants in one or more genetic markers in a sample is also useful in the present invention. 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 Table 1 (e.g., a NOD2/CARD15 gene, an IL12/IL23 pathway gene, *etc.*). Preferably, the presence or absence of at least one allelic variant, e.g., a single nucleotide polymorphism (SNP), in the NOD2/CARD15 gene and/or one or more genes in the IL12/IL23 pathway is determined. See, e.g., Barrett *et al.*, *Nat. Genet.*, 40:955-62 (2008) and Wang *et al.*, *Amer. J. Hum. Genet.*, 84:399-405 (2009).

25 **Table 1**

Gene	SNP
NOD2 (R702W) – SNP8	rs2066844
NOD2 (G908R) – SNP12	rs2066845
NOD2 (3020insC) – SNP13	rs5743293
ATG16L1 (T300A)	rs2241880
IL23R (R381Q)	rs11209026
DLG5	rs2165047

NOD2/CARD15	rs2066847
IL23R	rs11465804
ATG16L1	rs3828309
MST1	rs3197999
PTGER4	rs4613763
IRGM	rs11747270
TNFSF15	rs4263839
ZNF365	rs10995271
NKX2-3	rs11190140
PTPN2	rs2542151
PTPN22	rs2476601
ITLN1	rs2274910
IL12B	rs10045431
CDKAL1	rs6908425
CCR6	rs2301436
JAK2	rs10758669
C11orf30	rs7927894
LRRK2, MUC19	rs11175593
ORMDL3	rs2872507
STAT3	rs744166
ICOSLG	rs762421
GCKR	rs780094
BTNL2, SLC26A3, HLA-DRB1, HLA-DQA1	rs3763313
PUS10	rs13003464
CCL2, CCL7	rs991804
LYRM4	rs12529198
SLC22A23	rs17309827
IL18RAP	rs917997
IL12RB2	rs7546245
IL12RB1	rs374326
CD3D	rs3212262
CD3G	rs3212262
CD247	rs704853
JUN	rs6661505
CD3E	rs7937334
IL18R1	rs1035127
CCR5	
MAPK14	rs2237093

IL18	rs11214108
IFNG	rs10878698
MAP2K6	rs2905443
STAT4	rs1584945
IL12A	rs6800657
TYK2	rs12720356
ETV5	rs9867846
MAPK8	rs17697885
IRGM	rs13361189
IRGM	rs4958847
IRGM	rs1000113
IRGM	rs11747270
TL1A/TNFSF15	rs6478109
TL1A/TNFSF15	rs6478108
TL1A/TNFSF15	rs4263839
PTN22	rs2476601
CCR6	rs1456893
CCR6	rs2301436
5p13/PTGER4	rs1373692
5p13/PTGER4	rs4495224
5p13/PTGER4	rs7720838
5p13/PTGER4	rs4613763
ITLN1	rs2274910
ITLN1	rs9286879
ITLN1	rs11584383
IBD5/5q31	rs2188962
IBD5/5q31	rs252057
IBD5/5q31	rs10067603
GCKR	rs780094
TNFRSF6B	rs1736135
ZNF365	rs224136
ZNF365	rs10995271
C11orf30	rs7927894
LRRK2;MUC19	rs1175593
IL-27	rs8049439
TLR2	rs4696480
TLR2	rs3804099
TLR2	rs3804100
TLR2	rs5743704

TLR2	rs2405432
TLR4 (D299G)	rs4986790
TLR4 (T399I)	rs4986791
TLR4 (S360N)	rs4987233
TLR9	rs187084
TLR9	rs352140
NFC4	rs4821544
KIF21B	rs11584383
IKZF1	rs1456893
C11orf30	rs7927894
CCL2,CCL7	rs991804
ICOSLG	rs762421
TNFAIP3	rs7753394
FLJ45139	rs2836754
PTGER4	rs4613763
ECM1	rs7511649
ECM1 (T130M)	rs3737240
ECM1 (G290S)	rs13294
GLI1 (G933D)	rs2228224
GLI1 (Q1100E)	rs2228226
MDR1 (3435C>T)	rs1045642
MDR1 (A893S/T)	rs2032582
MAGI2	rs6962966
MAGI2	rs2160322
IL26	rs12815372
IFNG,IL26	rs1558744
IFNG,IL26	rs971545
IL26	rs2870946
ARPC2	rs12612347
IL10,IL19	rs3024493
IL10,IL19	rs3024505
IL23R	rs1004819
IL23R	rs2201841
IL23R	rs11465804
IL23R	rs10889677
BTLN2	rs9268480
HLA-DRB1	rs660895
MEP1	rs6920863
MEP1	rs2274658

MEP1	rs4714952
MEP1	rs1059276
PUS10	rs13003464
PUS10	rs6706689
RNF186	rs3806308
RNF186	rs1317209
RNF186	rs6426833
FCGR2A,C	rs10800309
CEP72	rs4957048
DLD,LAMB1	rs4598195
CAPN10,KIF1A	rs4676410
IL23R	rs11805303
IL23R	rs7517847
IL12B/p40	rs1368438
IL12B/p40	rs10045431
IL12B/p40	rs6556416
IL12B/p40	rs6887695
IL12B/p40	rs3212227
STAT3	rs744166
JAK2	rs10974914
JAK2	rs10758669
NKX2-3	rs6584283
NKX2-3	rs10883365
NKX2-3	rs11190140
IL18RAP	rs917997
LYRM4	rs12529198
CDKAL1	rs6908425
MAGI2	rs2160322
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

[0157] Additional SNPs useful in the present 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.*, 5 40:955-62 (2008).

1. NOD2/CARD15

[0158] The determination of the presence or absence of allelic variants such as SNPs in the NOD2/CARD15 gene is particularly useful in the present invention. As used herein, the term “NOD2/CARD15 variant” or “NOD2 variant” includes a nucleotide sequence of a 10 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 15 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 20 (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 Annesse *et al.*, *Eur. J. Hum. Genet.*, 7:567-573 (1999).

[0159] The mRNA (coding) and polypeptide sequences of human NOD2 are set forth in, 25 *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.

[0160] 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.

[0161] 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 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.

[0162] In some embodiments, a NOD2 variant is 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 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)).

[0163] A NOD2 variant useful in the 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 method of the invention is 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 acid 702. 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.

[0164] 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.

[0165] 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 response to bacterial lipopolysaccharides (Ogura *et al.*, *supra*). As used herein, the term “1007fs” includes a single nucleotide polymorphism within exon 11 of the NOD2 gene, which occurs in a triplet encoding amino acid 1007 of the NOD2 protein. The 1007fs variant contains a cytosine which has been added at position 121,139 of the AC007728 sequence, resulting in a frame shift mutation at amino acid 1007. Accordingly, this NOD2

variant is denoted “1007fs” and can also be denoted “3020insC” or “980fs” based on the earlier numbering system of Hugot *et al.*, *supra*. In addition, the 1007fs NOD2 variant is also known as the “SNP 13” allele or a “2” allele at SNP 13. The NCBI SNP ID number for 1007fs or SNP 13 is rs2066847.

- 5 **[0166]** One skilled in the art recognizes that a particular NOD2 variant allele or other polymorphic allele can be conveniently defined, for example, in comparison to a Centre d’Etude du Polymorphisme Humain (CEPH) reference individual such as the individual designated 1347-02 (Dib *et al.*, *Nature*, 380:152-154 (1996)), using commercially available reference DNA obtained, for example, from PE Biosystems (Foster City, CA).
- 10 In addition, specific information on SNPs can be obtained from the dbSNP of the National Center for Biotechnology Information (NCBI).

- [0167]** A NOD2 variant can also be located in a non-coding region of the NOD2 locus. Non-coding regions include, for example, intron sequences as well as 5’ and 3’ untranslated sequences. A non-limiting example of a NOD2 variant allele located in a
- 15 non-coding region of the NOD2 gene is the JW1 variant, which is described in Sugimura *et al.*, *Am. J. Hum. Genet.*, 72:509-518 (2003) and U.S. Patent Publication No. 20070072180. Examples of NOD2 variant alleles located in the 3’ untranslated region of the NOD2 gene include, without limitation, the JW15 and JW16 variant alleles, which are described in U.S. Patent Publication No. 20070072180. Examples of NOD2 variant alleles
- 20 located in the 5’ untranslated region (*e.g.*, promoter region) of the NOD2 gene include, without limitation, the JW17 and JW18 variant alleles, which are described in U.S. Patent Publication No. 20070072180.

- [0168]** As used herein, the term “JW1 variant allele” includes a genetic variation at nucleotide 158 of intervening sequence 8 (intron 8) of the NOD2 gene. In relation to the
- 25 AC007728 sequence, the JW1 variant allele is located at position 128,143. The genetic variation at nucleotide 158 of intron 8 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 of intron 8 has a cytosine at position 158. As non-limiting examples, a JW1 variant allele can have a cytosine (c) to adenine (a), cytosine (c)
- 30 to guanine (g), or cytosine (c) to thymine (t) substitution at nucleotide 158 of intron 8. In one embodiment, the JW1 variant allele is a change from a cytosine (c) to a thymine (t) at nucleotide 158 of NOD2 intron 8.

[0169] 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, the JW15 variant allele is a change from an adenine (a) to a cytosine (c) at nucleotide 118,790.

10 **[0170]** 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, the JW16 variant allele is a change from a guanine (g) to an adenine (a) at nucleotide 118,031.

[0171] 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, the JW17 variant allele is a change from a cytosine (c) to a thymine (t) at nucleotide 154,688.

[0172] 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, the JW18 variant allele is a change from a cytosine (c) to a thymine (t) at nucleotide 154,471.

[0173] It is understood that the methods of the invention can be practiced with these or
5 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.

10 VI. Examples

[0174] The present 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
15 essentially the same results.

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

[0175] This example illustrates a novel homogeneous assay for measuring anti-TNF α drug concentration in a patient sample (*e.g.*, serum) using size exclusion chromatography to detect the binding of the anti-TNF α drug to fluorescently labeled TNF α . The assay is
20 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-TNF α 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
25 solid surface such as an ELISA plate.

[0176] In one exemplary embodiment, TNF α is labeled with a fluorophore (*e.g.*, Alexa₆₄₇), wherein the fluorophore can be detected on either or both the visible and IR spectra. The labeled TNF α is incubated with human serum in a liquid phase reaction to allow the anti-TNF α drug present in the serum to bind. The labeled TNF α can also be
30 incubated with known amounts of the anti-TNF α 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-TNF α drug to the labeled TNF α results in a leftward shift of the peak compared to labeled TNF α alone. The concentration of the anti-TNF α drug present in the serum sample can then be compared to the standard curve and controls.

5 [0177] Figure 1 shows an example of the assay of the present invention wherein size exclusion HPLC is used to detect the binding between TNF α -Alexa₆₄₇ and HUMIRA™ (adalimumab). As shown in Figure 1, the binding of HUMIRA™ to TNF α -Alexa₆₄₇ caused a shift of the TNF α -Alexa₆₄₇ peak to the left.

[0178] Figure 2 shows dose response curves of HUMIRA™ binding to TNF α -Alexa₆₄₇.

10 In particular, Figure 2A shows that HUMIRA™ dose-dependently increased the shift of TNF α -Alexa₆₄₇ 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 TNF α -Alexa₆₄₇ 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 TNF α -
15 Alexa₆₄₇ in the size exclusion chromatography assay.

[0179] As such, this example demonstrates the utility of the present invention in monitoring patients receiving an anti-TNF α 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
20 treatment decisions, *e.g.*, whether to switch from the current anti-TNF α drug to a different TNF α inhibitor or to another type of therapy.

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

[0180] 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
25 exclusion chromatography to detect the binding of these autoantibodies to fluorescently labeled anti-TNF α 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
30 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.

- [0181]** The clinical utility of measuring autoantibodies (*e.g.*, HACA, HAHA, *etc.*) that are generated against TNF α 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-TNF α 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.
- [0182]** 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-TNF α drug (*e.g.*, with the bridging assay, HACA measurements must be taken at anti-TNF α 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 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.

[0183] In one exemplary embodiment, an anti-TNF α drug (*e.g.*, REMICADE™) is labeled with a fluorophore (*e.g.*, Alexa₆₄₇), wherein the fluorophore can be detected on either or both the visible and IR spectra. The labeled anti-TNF α 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-TNF α 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-TNF α 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-TNF α drug such as HUMIRA™.

[0184] The principle of this assay is based on the mobility shift of the antibody bound Alexa₆₄₇-labeled Remicade complex versus free Alexa₆₄₇-labeled Remicade on size exclusion- high performance liquid chromatography (SE-HPLC) due to the increase in molecular weight of the complex.

[0185] 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 μ L sample volume is loaded onto the column for each analysis.

[0186] The antibody bound Alexa₆₄₇-labeled Remicade complex is formed by incubating a known amount of the antibody and Alexa₆₄₇-labeled Remicade in the 1X PBS, pH 7.3, elution buffer at room temperature for 1 hour before SE-HPLC analysis.

[0187] Figure 5 shows a dose response analysis of anti-human IgG antibody binding to REMICADE™-Alexa₆₄₇ as detected using the size exclusion chromatography assay of the present invention. The binding of anti-IgG antibody to REMICADE™-Alexa₆₄₇ caused a shift of the REMICADE™-Alexa₆₄₇ peak to the left. Figure 6 shows a second dose response analysis of anti-human IgG antibody binding to REMICADE™-Alexa₆₄₇ 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™-Alexa₆₄₇ complexes, as indicated by a shift of the REMICADE™-Alexa₆₄₇ peak to the left. Figure 7 shows dose response curves of anti-IgG antibody binding to REMICADE™-Alexa₆₄₇.

[0188] Figure 8 shows REMICADE™-Alexa₆₄₇ 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™-Alexa₆₄₇ caused a shift of the REMICADE™-Alexa₆₄₇ 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™.

[0189] 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).

[0190] As such, this example demonstrates the utility of the present invention in monitoring patients receiving an anti-TNF α 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-TNF α drug that preclude further treatment with the drug.

[0191] In conclusion, Examples 1 and 2 demonstrate that TNF α and anti-TNF α antibodies can be efficiently labeled with Alexa₆₄₇. When labeled TNF α -Alexa₆₄₇ was incubated with anti-TNF α antibodies, the retention time of the labeled TNF α /anti-TNF α antibody complex was shifted, and the amount of anti-TNF α antibody that caused the shift

could be quantitated with HPLC. Furthermore, when labeled anti-TNF α antibody was incubated with anti-human IgG antibody, the retention time of the labeled anti-TNF α 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-TNF α antibody and HACA/HAHA assays and could be used to quantitate patient serum anti-TNF α antibody or HACA/HAHA levels. Advantageously, the present 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-TNF α 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-TNF α biologic drugs (*e.g.*, Remicade, Humira, Enbrel and Cimzia) as well as the neutralizing antibody (anti-RemicadeTM) in patient serum.

Example 3. Measurement of Human Anti-Chimeric Antibodies (HACA) and Infliximab (IFX) Levels in Patient Serum Using A Novel Mobility Shift Assay.

ABSTRACT

[0192] Background: Infliximab (IFX) is a chimeric monoclonal antibody therapeutic against TNF α that has been shown to be effective in treating autoimmune diseases such as rheumatoid arthritis (RA) and inflammatory bowel disease (IBD). However, antibodies against IFX were found in some IFX-treated patients through the detection of human anti-chimeric antibodies (HACA), which may reduce the drug's efficacy or induce adverse effects. Monitoring of HACA and IFX levels in individual patients may help to optimize the dosing and treatment with IFX. Current methods for detecting HACA are based on solid-phase assays, which are limited by the fact that the presence of IFX in the circulation may mask the presence of HACA and, therefore, measurement can only be done at least 8 weeks following a dose of IFX. Moreover, this time-lapse further confounds the assays because of the rapid clearance of the high molecular weight immune complexes in the blood circulation. To overcome these drawbacks, we have developed and evaluated a new method to measure serum IFX and HACA levels in patients treated with IFX.

- [0193] Methods:** A novel non-radiolabeled, liquid-phase, size-exclusion (SE)-HPLC mobility shift assay was developed to measure the HACA and IFX levels in serum from patients treated with IFX. The immuno-complex (*e.g.*, TNF α /IFX or IFX/HACA), free TNF α or IFX, and the ratio of bound/free can be resolved and calculated with high sensitivity. Serum concentrations of IFX or HACA were determined with standard curves generated by incubating with different concentrations of IFX or pooled HACA-positive serum. Using this novel assay, we have measured IFX and HACA levels in sera collected from IBD patients treated with IFX who had relapsed and compared the results with those obtained by the traditional Bridge ELISA assay.
- [0194] Results:** Dose-response curves were generated from the novel assay with high sensitivity. Detection of HACA was demonstrated in the presence of excess IFX. In the 117 serum samples from patients treated with IFX, 65 samples were found to have IFX levels above the detection limit and the average was 11.0 $\pm$ 6.9 mg/mL. For HACA levels, 33 (28.2%) samples were found to be positive while the Bridge ELISA assay detected only 24 positive samples. We also identified 9 false negatives and 9 false positives from the samples 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.
- [0195] 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

- [0196]** Tumor necrosis factor-alpha (TNF α) 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 TNF α with therapeutic antibodies such as Infliximab (human-murine chimeric monoclonal IgG1 κ) 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-TNF α 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, 5 many patients with secondary response failure to one anti-TNF α drug benefit from a switch to other anti-TNF α 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 10 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)).

[0197] 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 15 present 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 invention is a non-radiolabeled, liquid-phase, size-exclusion (SE)-HPLC assay that is 20 capable of measuring HACA and IFX levels in serum from patients while being treated with IFX.

[0198] The following are rationales for measuring the serum concentrations of anti-TNF α biologic drugs and antibodies against TNF α biologic drugs in patients: (1) for PK studies in clinical trials; (2) it may be required by the FDA during clinical trials to monitor 25 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

30 [0199] *SE-HPLC analysis of Infliximab (IFX) levels in patient serum.* Human recombinant TNF α was labeled with a fluorophore ("Fl" such as, *e.g.*, Alexa Fluor[®] 488) according to the manufacture's instructions. Labeled TNF α was incubated with different

amounts of IFX or patient serum for one hour at room temperature. Samples of 100 μ L volume were analyzed by size-exclusion chromatography on an HPLC system. FLD was used to monitor the free TNF α -FI and the bound TNF α -FI immuno-complex based on their retention times. Serum IFX levels were calculated from the standard curve.

5 **[0200]** *SE-HPLC analysis of HACA levels in patient serum.* Purified IFX was labeled with FI. 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 μ L volume were analyzed by size-exclusion chromatography on an HPLC system. FLD was used to monitor the free IFX-FI and the bound IFX-FI immuno-complex based on their
10 retention times. The ratio of bound and free IFX- FI was used to determine the HACA level.

[0201] *Mobility Shift Assay Procedure to Measure HACA in Serum.* The principle of this assay is based on the mobility shift of the HACA bound FI-labeled Infliximab (IFX) complex versus free FI-labeled IFX on size exclusion-high performance liquid
15 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 μ L sample volume is loaded onto the column for each
20 analysis. The HACA bound FI-labeled IFX complex is formed by incubating serum from IFX treated patient and FI-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- α , in order to validate the assay.

25 **RESULTS**

[0202] Figure 11 shows the separation of the HACA bound IFX-FI complex from the free IFX-FI 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-FI
30 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.

- [0203]** Figure 13 shows the separation of the IFX bound TNF α -FI complex from the free TNF α -FI 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 TNF α -FI remains in the chromatogram and the more the immuno-complex is formed. Figure 14 shows the dose-response curves of the TNF α -FI peak shift caused by the addition of IFX. Based on the added IFX, the detection limit is 10 ng/mL of IFX in serum.
- [0204]** 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 2). 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 μ g/ml. Table 3 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 2. 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 3. Patient Sample Analysis on Serum Levels of HACA with Bridge Assay (Cut Off 1.69 μ g/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.

5 **[0205]** 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
10 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.

[0206] 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 in
15 the following tables:

Infliximab assay		HACA assay	
Parameter	CV%	Parameter	CV%
Intra-assay Precision	2.89	Intra-assay Precision	3.96
Inter-assay Precision		Inter-assay Precision	
Run to Run	4.57	Run to Run	4.15
Analyst to Analyst	6.06	Analyst to Analyst	5.84
Instrument to Instrument	2.73	Instrument to Instrument	6.88

Infliximab assay			
Interference Agent	Typical Range	Concentration tested	Interference
IgG, IgA, IgM	0.4-16 mg/mL	10, 2.0, 1.5 mg/mL	NA
ATI	3.71-150 U/mL (0-60 µg/mL)	100 U/mL (~ 55 µg/mL)	Interferes with detection of low concentration IFX samples (<5 µg/mL)
Rheumatoid Factor	>30 IU/mL (RA positive patients)	Up to 387 IU/mL	NA
TNF-α	6.2-6.6 pg/mL	0.0125 ng/mL – 40 µg/mL	100 ng/mL
TNFR1/TNFR2	1.9/4.5 ng/mL	0.1-1000 ng/mL	NA
Hemolyzed Serum	>20 HI	100-300 HI	NA
The following agents were also tested and did not show interference: Azathioprine, Methotrexate, TNF-β, Lipemic serum, Hemoglobin			

HACA assay

Interference Agent	Typical Range	Concentration tested	Interference
IgG, IgA, IgM	0.4-16 mg/mL	10, 2.0, 1.5 mg/mL	NA
Infliximab	0-100 µg/mL	0.78-100 µg/mL	NA
Rheumatoid Factor	>30 IU/mL (RA positive patients)	Up to 774 IU/mL	NA
TNF-α	6.2-6.6 pg/mL	0.0125 ng/mL – 40 µg/mL	250 ng/mL
TNFR1/TNFR2	1.9/4.5 ng/mL	0.1-1000 ng/mL	NA
Hemolyzed Serum	>20 HI	100-300 HI	NA
The following agents were also tested and did not show interference: Azathioprine, Methotrexate, TNF-β, Lipemic serum, Hemoglobin			

CONCLUSION

[0207] Anti-TNFα biologic drugs can be readily labeled with a fluorophore (“Fl”) and the mobility shift assay format used for measuring HACA/HAHA is a homogeneous assay without the coating of antigens to a solid surface and multiple washing and incubation steps like a typical ELISA. Incubation of Fl-labeled IFX with HACA-positive serum results in the formation of an immune complex which elutes at a different position compared to free Fl-labeled IFX in SE-HPLC and thus the amount of HACA can be quantitated. The presence of 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.

[0208] 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.

- 5 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
10 **Anti-Chimeric Antibodies (HACA) in Patient Serum Using Novel Mobility Shift**
Assays.

- [0209] 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
15 exclusion chromatography to detect the binding of these autoantibodies to fluorescently labeled anti-TNF α drug in the presence of fluorescently labeled TNF α . 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
20 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.

- [0210] In one exemplary embodiment, an anti-TNF α drug (*e.g.*, REMICADE™) is
25 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, TNF α 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-TNF α drug is incubated with human serum in a liquid
30 phase reaction and the labeled TNF α is added to the reaction to allow the formation of complexes (*i.e.*, immuno-complexes) between the labeled anti-TNF α drug, labeled TNF α , 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 TNF α to the labeled anti-TNF α 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-TNF α drug (*e.g.*, “Immuno-Complex 2” in Figure 17A), the labeled drug alone, or the labeled TNF α alone. The presence of this ternary complex of autoantibody (*e.g.*, HACA), labeled TNF α , and labeled anti-TNF α 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-TNF α antibody and TNF α . In one particular 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-TNF α , 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-TNF α peaks. However, the presence of the binary complex between the autoantibody (*e.g.*, HACA) and the labeled anti-TNF α drug (*e.g.*, “Immuno-Complex 2” in Figure 17B) in the absence of the ternary complex of autoantibody (*e.g.*, HACA), labeled TNF α , and labeled anti-TNF α 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-TNF α antibody and TNF α . In one particular 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-TNF α peak. In certain instances, the presence of neutralizing HACA indicates that the current therapy with REMICADE™ should be switched to another anti-TNF α drug such as HUMIRA™.

[0211] In an alternative embodiment, the labeled anti-TNF α 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-TNF α drug and HACA present in the serum. Following incubation, the samples are loaded directly onto a first size exclusion column. Binding of the autoantibody (*e.g.*, HACA) to the labeled anti-TNF α drug results in a leftward shift of the peak (*e.g.*, “Immuno-Complex 2” in Figure 18) compared to the labeled drug alone. The labeled TNF α is then added to the reaction to determine whether it is capable of displacing (*e.g.*, competing with) the autoantibody (*e.g.*, HACA) for binding to the labeled anti-TNF α drug, to thereby allow the formation of complexes (*i.e.*, immuno-complexes) between the labeled anti-TNF α drug and the labeled TNF α .

Following incubation, the samples are loaded directly onto a second size exclusion column. Binding of the labeled anti-TNF α drug to the labeled TNF α results in a leftward shift of the peak (*e.g.*, “Immuno-Complex 3” in Figure 18) compared to the labeled TNF α alone. Disruption of the binding between the autoantibody (*e.g.*, HACA) and the labeled anti-TNF α drug by the addition of the labeled TNF α 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-TNF α antibody and TNF α . In certain instances, the presence of neutralizing HACA indicates that the current therapy with REMICADE™ should be switched to another anti-TNF α drug such as HUMIRA™.

Example 5. Analysis of Human Anti-Drug Antibodies (ADA) to Adalimumab in Patient Serum Using a Novel Homogeneous Mobility Shift Assay.

[0212] Background and Aim: Monoclonal antibodies against TNF- α such as infliximab (IFX), adalimumab (HUMIRA™), and certolizumab have been shown to be effective in treating inflammatory bowel disease (IBD) and other inflammatory disorders. Anti-drug antibodies (ADA) may reduce the drug’s efficacy and/or induce adverse effects. However, ADAs have been found not only in patients treated with the chimeric antibody infliximab, but also in patients treated with the humanized antibody adalimumab. Monitoring of ADA and drug levels in individual patients may help optimize treatment and dosing of the patient. We have developed a non-radio labeled liquid-phase homogeneous mobility shift assay to accurately measure in the serum both HACA (Human Anti-Chimeric Antibody) and IFX from patients. This assay method overcomes a major limitation of the current solid-phase assays for detecting HACA, namely the inability to accurately detect HACA in the presence of IFX in circulation. In the present study, we have evaluated this new method for measuring serum ADA and drug levels in patients treated with the humanized antibody drug, adalimumab.

[0213] 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.

5 **[0214] 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
10 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.

[0215] Conclusions: The non-radio labeled liquid-phase homogeneous mobility shift assay of the present invention has been applied to measure ADA and adalimumab levels in
15 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.

20 **ABSTRACT**

[0216] 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
25 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 Antibody) and IFX from IFX-treated patients. This assay overcomes the major limitation of the current solid-phase assays for detecting HACA,
30 namely the inability to accurately detect HACA in the presence of IFX in circulation. In the present study, we have evaluated this new assay to measure serum ADA and drug levels in patients treated with the fully human antibody drug, ADL.

- [0217] **Methods:** The mobility shift assay was based on the shift in retention time of the antigen-antibody immunocomplex versus free antigen on size-exclusion chromatography. Fluorophore-labeled ADL or TNF- α and internal control were mixed with serum samples to measure the mobility shift of labeled ADL and TNF- α in the presence of ADA or drug.
- 5 The changes in the ratio of free ADL or TNF- α to internal control are the indicators of the immunocomplex formation. Serum concentrations of ADA or ADL were determined with standard curves generated by incubating with different concentrations of anti-human IgG antibody or purified ADL. Using this assay, we measured ADL and ADA levels in sera collected from IBD patients treated with ADL.
- 10 [0218] **Results:** Dose-response curves were generated with anti-human IgG antibody for the measurement of mobility shift of labeled ADL. The detection limit of the assay was 10 ng of anti-human IgG. Sera from 100 healthy controls were tested for the ADA and all of the samples had an ADA level below detection limit (no shift of free labeled ADL). Detection of ADA was demonstrated in five out of 114 IBD patient samples
- 15 treated with ADL. To measure the drug concentration in patients treated with ADL, we generated a standard curve with different amounts of ADL on the shift of labeled TNF- α with the detection limit of 10 ng.
- [0219] **Conclusions:** We have applied our proprietary non-radio labeled liquid-phase homogeneous mobility shift assay to measure the ADA and ADL levels in serum from
- 20 patients treated with ADL. The assays are reproducible with high sensitivity and accuracy, and are useful for evaluating ADA levels in serum samples from patients treated with ADL.

INTRODUCTION

- [0220] Anti-tumor necrosis factor-alpha (TNF- α) biologics such as infliximab (IFX),
- 25 etanercept, adalimumab (ADL) and certolizumab pegol have been shown to reduce disease activity in a number of autoimmune diseases, including Crohn's Disease (CD) and rheumatoid arthritis (RA). However, some patients do not respond to anti-TNF- α therapy, while others need higher or more frequent dosage due to lack of sufficient response, or develop infusion reactions.
- 30 [0221] Immunogenicity of therapeutic antibodies which causes the patients to develop antibodies against the drugs may contribute to the failure of the treatments and infusion reactions. Chimeric antibodies like IFX have a higher potential of inducing antibody

generation compared to fully humanized antibodies such as ADL. The prevalence of antibodies to IFX (HACA) in RA patients varies from 12% to 44% and seems to be inversely proportional to the level of IFX in patient serum and therapeutic response. While the fully humanized ADL is supposed to be less immunogenic than murine or chimeric antibodies, several studies have reported the formation of human anti-humanized antibodies (HAHA) and showed the prevalence of antibody generation from 1% to 87% in RA and CD patients (Aikawa *et al.*, Immunogenicity of Anti-TNF-alpha agents in autoimmune diseases. *Clin. Rev. Allergy Immunol.*, 38(2-3):82-9 (2010)).

- 5 antibodies, several studies have reported the formation of human anti-humanized antibodies (HAHA) and showed the prevalence of antibody generation from 1% to 87% in RA and CD patients (Aikawa *et al.*, Immunogenicity of Anti-TNF-alpha agents in autoimmune diseases. *Clin. Rev. Allergy Immunol.*, 38(2-3):82-9 (2010)).
- 10 [0222] Many patients with secondary response failure to one anti-TNF- α drug may benefit from switching to another anti-TNF- α drug or increasing dosage and/or dosing frequency. Monitoring of patients for drug and anti-drug antibody (ADA) levels is therefore warranted so that drug administration can be tailored to the individual patient. This approach allows dose adjustment when warranted or cessation of medication when ADA levels are present. (Bendtzen *et al.*, Individual medicine in inflammatory bowel disease: monitoring bioavailability, pharmacokinetics and immunogenicity of anti-tumour necrosis factor-alpha antibodies. *Scand. J. Gastroenterol.*, 44(7):774-81 (2009); Afif *et al.*, Clinical utility of measuring infliximab and human anti-chimeric antibody concentrations in patients with inflammatory bowel disease. *Am. J. Gastroenterol.*, 105(5):1133-9 (2010)).
- 15
- 20 [0223] A number of assays have been developed to measure HACA and HAHA. One of the limitations of the current methodologies is that ADA levels cannot be reliably measured when there is a high level of drugs in the circulation.

- [0224] We have developed a proprietary non-radiolabeled, liquid-phase, mobility shift assay to measure the ADA and ADL levels in serum from patients treated with ADL which is not affected by the presence of the drug in the serum.
- 25

METHODS

- [0225] Fluorophore (FI)-labeled ADL was incubated with patient serum to form the immunocomplex. A FI-labeled small peptide was included as an internal control in each reaction. Different amounts of anti-human IgG were used to generate a standard curve to determine the serum ADA level. Free FI-labeled ADL was separated from the antibody bound complex based on its molecular weight by size-exclusion chromatography. The ratio of free FI-labeled ADL to internal control from each sample was used to extrapolate
- 30

the HAHA concentration from the standard curve. A similar methodology was used to measure ADL levels in patient serum samples with FI-labeled TNF- α .

RESULTS

- 5 **[0226]** Figure 19 shows the separation of the anti-human IgG bound FI-ADL complex from the free FI-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.
- 10 **[0227]** 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 “FI-BioCyt” corresponds to an Alexa Fluor[®] 488-biotin (BioCyt) which combines the green-
15 fluorescent Alexa Fluor[®] 488 fluorophore with biotin and an aldehyde-fixable primary amine (lysine) (Invitrogen Corp.; Carlsbad, CA).
- [0228]** Figure 21 shows the separation of the ADL bound TNF- α -FI complex from the free TNF- α -FI 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
20 min. The more the ADL is added in the reaction mixture, the less the free TNF- α -FI peak remains in the chromatogram and the more the immuno-complex is formed.
- [0229]** Figure 22 shows the dose-response curves of the TNF- α -FI peak shift caused by the addition of ADL. Based on the added ADL, the detection limit is 10 ng/mL of ADL in serum.
- 25 **[0230]** Table 4 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 FI-ADL), whereas 5 out of the 114 patient samples had an ADA concentration of 0.012 to >20 μ g/ml. The mean of ADL levels in 100 healthy
30 subject samples was 0.76 ± 1.0 μ g/ml (range 0 to 9.4 μ g/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.

Table 4. Patient Serum Levels of ADA and ADL Measured by the Mobility Shift Assay

	Subjects (n)	Sex (M/F)	Age (Years) (Mean)	ADA Positive	ADL level (µg/ml)
Healthy Control	100	38/62	18-62 (37.1)	0	0.76±1.00
IBD Patient Treated with ADL	114	51/63	20-69 (39.9)	5 (4.3%)	10.80±17.80

CONCLUSIONS

- [0231] The mobility shift assay format used for measuring HACA/IFX is a homogeneous assay without the coating of antigens to a solid surface, and without multiple washing and incubation steps like a typical ELISA. This assay can be applied to measure ADA and anti-TNF drugs. The sensitivity of the assay (in µg/ml range) is higher for both ADA and ADL measurement with patient serum compared to ELISA methods (in mg/ml range). Healthy control serum samples did not cause mobility shift of the FI-labeled ADL, and 4.3% of the patients treated with ADL were found to have ADA by this assay.
- Although healthy control serum samples caused mobility shift of the FI-labeled TNF-α, which may have been due to the presence of soluble free receptor of TNF-α, the average of ADL in patients treated with ADL was much higher (10.8 vs. 0.76 mg/ml). Early detection of ADA and monitoring of ADL drug level while the patient is receiving ADL treatment will allow the physician to optimize the dosing of ADL or switch to another anti-TNF-α drug when appropriate and, thereby, optimizing the overall management of the patient's disease.

Table 5. Patient Serum Levels of ADA and ADL Measured by the Mobility Shift Assay

	Subjects (n)	Sex (M/F)	Age (Mean)	ADL Level (µg/ml)	ADA Positive
Healthy Control	100	38/62	18-62 (37.1)	0.76 ± 1.00	0
IBD Patient Treated with ADL	114	51/63	20-69 (39.9)	10.80 ± 17.80	0-4 µg/ml ADL: 4 of 42 (9.52%)

- Using this mobility shift assay we analyzed serum samples from 100 healthy subjects, and 114 IBD patients treated with ADL, for ADA and ADL levels. All 100 healthy subject samples had ADA levels below the limit of detection (no shift of the free FI-ADL), whereas 4 out of the 42 patient samples with 0-4µg/mL ADL had an average ADA concentration of 0.012 to >20 µg/ml. Mean ADL levels in 100 healthy subject samples was 0.76±1.0 mg/ml (range 0 to 9.4 mg/ml). Mean ADL levels in 114 serum samples from patients treated with ADL was 10.8±17.8 mg/ml (range 0 – 139 mg/ml). Four out of four ADA positive samples had undetectable levels of ADL. For the detection of ADA, the 114 IBD patients treated with ADL were divided into two categories, 0-4µg/ml of ADL and >4µg/ml of ADL. Patients with greater than 4µg/ml of ADL will be tested with a larger amount of ADL-FI to address the competition of circulating ADL with ADL-FI.

[0232] Healthy control serum samples do not cause mobility shift of the FI-labeled ADL. In a preliminary study, 9.52% of patients with 0.4 µg/ml ADL were found to have ADA in this assay. Further evaluation of normal samples and patients samples with higher concentrations of ADL-FI will be done.

Example 7. Combinatorial Algorithm for Optimizing Anti-TNF Drug Therapy.

[0233] This example describes methods for optimizing therapy, reducing toxicity, and/or monitoring the efficacy of therapeutic treatment by applying a statistical algorithm such as, *e.g.*, a learning statistical classifier system, to one or more (*e.g.*, a combination of two, three, four, five, six, seven, or more) biochemical markers, serological markers, and/or genetic markers. Accordingly, the methods set forth in the present example provide information useful for guiding treatment decisions for patients receiving anti-TNF drug therapy, *e.g.*, by determining when or how to adjust or modify (*e.g.*, increase or decrease) the subsequent dose of an anti-TNF drug, by determining when or how to combine an anti-TNF 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-TNF drug).

[0234] As a non-limiting example, the presence, level, or genotype of one, two, three, four, five, or all six of the following classes of biochemical markers, serological markers, and/or genetic markers can be detected, measured, or determined in a patient sample (*e.g.*, a serum sample from a patient on anti-TNF drug therapy):

- (1) anti-TNF drug levels (*e.g.*, levels of free anti-TNFα therapeutic antibody);
- (2) anti-drug antibody (ADA) levels (*e.g.*, level of autoantibody to the anti-TNF drug);
- (3) TNFα levels;
- (4) levels of one, two, three, four, five, six, seven, or more additional cytokines (*e.g.*, IL-6, IL-1β, IFN-γ, IL-10, *etc.*) and/or markers for other mechanisms of

inflammation (*e.g.*, inflammatory markers such as CRP, SAA, ICAM-1, and/or VCAM-1);

- 5 (5) the presence or absence of one or more mutations in one or more genetic markers such as inflammatory pathway genes, *e.g.*, the presence or absence of variant alleles (*e.g.*, SNPs) in one or more inflammatory markers such as, *e.g.*, NOD2/CARD15 (*e.g.*, SNP 8, SNP 12, and/or SNP 13 described in US Patent No. 7,592,437), ATG16L1 (*e.g.*, the rs2241880 (T300A) SNP described in Lakatos *et al.*, *Digestive and Liver Disease*, 40 (2008) 867-873), IL23R (*e.g.*, the rs11209026 (R381Q) SNP described in Lakatos *et al.*), the human leukocyte antigen (HLA) genes and/or cytokine genes described in, *e.g.*, Gasche *et al.* (*Eur. J. Gastroenterology & Hepatology*, (2003) 15:599-606), and the DLG5 and/or OCTN genes from the IBD5 locus;
- 10 (6) levels of one or more of the above biochemical markers and/or serological markers at multiple time points (*e.g.*, at 28 weeks, 60 weeks, *etc.*); and
- 15 (7) combinations thereof.

[0235] A single statistical algorithm or a combination of two or more statistical algorithms described herein can then be applied to the presence, concentration level, or genotype of the markers detected, measured, or determined in the sample to thereby optimize therapy, reduce toxicity, or monitor the efficacy of therapeutic treatment with the anti-TNF drug. As such, the methods described in this example find utility in determining patient management by determining patient immune status.

20

[0236] Table 6 below provides a summary of cytokine levels in normal and patient samples.

Table 6

Sample		IFN- γ	IL-1 β	IL-6	TNF- α
Normal Control n=64	Mean $\pm$ SD (pg/mL)	0.88 $\pm$ 2.71	0.17 $\pm$ 0.72	0.65 $\pm$ 0.58	1.78 $\pm$ 0.71
	Range (pg/mL)	0.00-141.42	0.00-44.41	0.12-31.70	0.35-73.83
	Mean $\pm$ 2SD (pg/mL)	5.3	1.6	1.82	3.19
	Positive/ Total	5/64	2/64	11/64	3/64
UC Samples (IFX treated) n=43	Mean $\pm$ SD (pg/mL)	1.96 $\pm$ 2.24	0.13 $\pm$ 0.08	0.85 $\pm$ 1.23	6.08 $\pm$ 6.54
	Range (pg/mL)	0.00-9.34	0.01-0.47	0.12-5.60	0.13-35.54
	Positive/ Total	3/43 (7.0%)	0/43 (0.0%)	5/43 (11.6%)	34/43 (79.1%)
Humira Treated CD samples n=117	Mean $\pm$ SD (pg/mL)	2.88 $\pm$ 1.25	0.59 $\pm$ 1.22	1.56 $\pm$ 1.55	8.03 $\pm$ 21.10
	Range (pg/mL)	0.00-36.29	0.00-8.59	0.17-11.93	0.49-207.29
	Positive/ Total	14/117 (12.0%)	11/117 (9.4%)	35/117 (29.9%)	67/117 (57.3%)
HACA positive samples n=94	Mean $\pm$ SD (pg/mL)	6.21 $\pm$ 12.12	1.36 $\pm$ 37.7	3.58 $\pm$ 6.35	21.16 $\pm$ 32.78
	Range (pg/mL)	0.18-98.87	0-365.11	0.09-2302.4	0.09-176.12
	Positive/ Total	25/94 (26.60%)	4/94 (4.3%)	39/94 (41.5%)	62/94 (66.0%)

[0237] 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 presence, level, and/or genotype of one or more biochemical markers, serological markers, and/or genetic markers as described herein. In each of the following scenarios, one, two, or more statistical algorithms can then be applied to optimize therapy and/or reduce toxicity associated with the anti-TNF drug.

Scenario #1: High level of anti-TNF drug with low level of anti-drug antibody (ADA) and low levels of inflammatory cytokines.

[0238] Drug levels = 10-50 ng/10 μ l; ADA levels = 0.1-2 ng/10 μ l; TNF α levels = 1-8 pg/ml; IL-6 levels = 0.1-3 pg/ml; IL-1 β levels = 0.0-2 pg/ml; IFN- γ levels = 0-6 pg/ml; antibody against IL-10 not detected. Patient samples having this marker profile include samples from patients BAB and JAA on visit 10 ("V10"). *See*, Figure 16b.

[0239] Patients receiving anti-TNF drug therapy and having this particular marker profile should be treated with immunosuppressive drugs like azathioprine along with the anti-TNF drug (*e.g.*, infliximab).

Scenario #2: Medium level of anti-TNF drug with low level of ADA and low levels of inflammatory cytokines.

[0240] Drug levels = 5-20 ng/10µl; ADA levels = 0.1-2 ng/10µl; TNFα levels = 1-8 pg/ml; IL-6 levels = 0.1–3 pg/ml; IL-1β levels = 0.0-2 pg/ml; IFN-γ levels = 0-6 pg/ml; antibody against IL-10 not detected. Patient samples having this marker profile include samples from patients DGO, JAG, and JJH on visit 10 (“V10”). *See*, Figure 16b.

- 5 [0241] Patients receiving anti-TNF drug therapy and having this particular marker profile should be treated with immunosuppressive drugs like azathioprine along with a higher dose of the anti-TNF drug (*e.g.*, infliximab). One skilled in the art will know of suitable higher or lower doses to which the current course of therapy can be adjusted such that drug therapy is optimized, *e.g.*, a subsequent dose that is at least about 1.5, 2, 2.5, 3,
10 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 15, 20, 25, 30, 35, 40, 45, 50, or 100-fold higher or lower than the current dose.

Scenario #3: Medium level of anti-TNF drug with medium level of ADA and low levels of inflammatory cytokines.

- [0242] Drug levels = 5-20 ng/10µl; ADA levels = 0.5-10 ng/10 µl; TNFα levels = 1-8
15 pg/ml; IL-6 levels = 0.1-3 pg/ml; IL-1β levels = 0.0-2 pg/ml; IFN-γ levels = 0-6 pg/ml; antibody against IL-10 not detected. Patient samples having this marker profile include samples from patient JMM on visit 10 (“V10”) and patient J-L on visit 14 (“V14”). *See*, Figure 16b.

- [0243] Patients receiving anti-TNF drug therapy and having this particular marker
20 profile should be treated with a different drug. As a non-limiting example, a patient on infliximab (IFX) therapy and having medium levels of IFX and ADA (*i.e.*, HACA) with low levels of inflammatory cytokines should be switched to therapy with adalimumab (HUMIRA™).

- Scenario #4: Low level of anti-TNF drug with high level of ADA and high levels of
25 inflammatory cytokines.

[0244] Drug levels: 0-5 ng/10 µl; ADA levels = 3.0-50 ng/10 µl; TNFα levels = 10-60 pg/ml; IL-6 levels = 0.1-50 pg/ml; IL-1β levels = 0.0-366 pg/ml; IFN-γ levels = 0.15-100 pg/ml; antibody against IL-10 not detected. Patient samples having this marker profile include samples from all patients on visit 14 (“V14”) in Figure 16b.

- 30 [0245] Patients receiving anti-TNF drug therapy and having this particular marker profile should be treated with a different drug. As a non-limiting example, a patient on

infliximab (IFX) therapy and having a low level of IFX with high levels of ADA (*i.e.*, HACA) and inflammatory cytokines should be switched to therapy with adalimumab (HUMIRA™).

Scenario #5: High levels of inflammatory cytokines.

5 [0246] High TNF α levels (*e.g.*, 10-60 pg/ml); high IL-6 levels (*e.g.*, 0.1-50 pg/ml); high IL-1 β levels (*e.g.*, 0.0-366 pg/ml); high IFN- γ levels (*e.g.*, 0.15-100 pg/ml); high levels of other inflammatory molecules; +/- antibodies against anti-inflammatory cytokines (*e.g.*, antibody against IL-10 either detected or not detected).

[0247] Patients receiving anti-TNF drug therapy and having this particular marker
10 profile should either be treated with a different drug or treated with immunosuppressive drugs like methotrexate (MTX) or azathioprine (AZA) along with a higher dose of the anti-TNF drug. In particular, this marker profile of high levels of inflammatory cytokines from a patient on infliximab (IFX) therapy indicates that the current course of therapy is not working and the patient should be switched to therapy with adalimumab (HUMIRA™)
15 or should be treated with one or more immunosuppressive drugs along with a higher dose of IFX.

Example 8. Determination of Different Anti-Drug Antibody (ADA) Isotypes.

[0248] This example describes the determination of different anti-drug antibody (ADA) isotypes in ADA-positive patients receiving anti-TNF drug therapy. Non-limiting
20 examples of antibody isotypes include IgA, IgD, IgE, IgG, and IgM. In certain aspects, the detection of the presence or level of a specific ADA isotype or a particular combination of ADA isotypes is associated with different clinical outcomes.

[0249] Figure 23 shows the elution times of different ADA isotypes in HACA-positive patient serum. In particular embodiments, the therapeutic implications of anti-TNF drug
25 therapy can be determined based upon whether the ADA is an IgM isotype (retention time: ~6.5-7.5 minutes), IgA isotype (7.5-8.5 minutes), or IgG isotype (8.5-9.5 minutes) based on size and appropriate dilution experiments. The retention times for labeled anti-TNF drugs such as Humira and Remicade is about 10.8 minutes, and the retention time for the internal control (IC) is about 13.5 minutes.

30 [0250] In one study, 200 patient samples were assayed in accordance with the methods of the present invention for detecting ADA by size exclusion chromatography. Samples 1-

100 were controls (*e.g.*, normal healthy control samples), while samples 101-200 were obtained from patients receiving Remicade therapy and were HACA-positive by the Bridge assay. An unadjusted plot of signal intensity (Y-axis) versus elution time (X-axis) was generated for all 200 samples. The following three adjustments were then made to the
5 plot: (1) Standardized Y (low) to the baseline on the left; (2) Standardized Y (high) to the control peak on the right; and (3) Standardized X to the control peak on the right.

[0251] The data from all 200 samples were split into the 100 control samples and the 100 HACA-positive samples and then averaged. Figure 24 shows a plot of the mean of the control samples. Figure 25 shows a plot of the mean of the positive samples. Notably, a
10 comparison of the plots in Figures 24 and 25 shows a decrease in the signal intensity for labeled Remicade (“Primary Peak”) and the appearance of two distinct ADA isotype peaks, corresponding to HACA IgA (“A peak”) and HACA IgG (“G peak”).

[0252] Figure 26 illustrates a side-by-side comparison of the Primary Peak signals (*i.e.*, corresponding to labeled Remicade signal intensity) for the 100 control samples and the
15 100 positive samples. Values shown are average values from elution times 9.71 to 12.23 minutes. In particular, Figure 26 shows decrease in the signal intensity for labeled Remicade for the 100 HACA-positive samples, which correlates to the formation of complexes between the labeled Remicade and HACA. Figure 26 also shows that a number of positive samples had a high level of HACA, as evidenced by the low Primary Peak
20 signals (*e.g.*, between 0 and 0.2 on the X-axis). Figure 27 shows a Receiver Operating Characteristic (ROC) curve for the Primary Peak data from Figure 26. The Area Under the Curve (AUC) was 0.986.

[0253] Next, the average values for the Primary Peak versus the sum of the other peaks (IgG, IgA, and IgM) were plotted. Figure 28 shows a plot of Non-primary peaks on the X-
25 axis (which correspond to the sum of the IgG, IgA, and IgM peaks) versus the Primary Peak on the Y-axis for the 100 HACA-positive samples. The linear relationship illustrated in this plot may be used as an assay check (*i.e.*, an assay control). Figure 29 shows a plot of the IgG versus IgA versus IgM peaks for all 200 samples.

Example 9. Proximity-Based Determination of Different ADA Isotypes.

30 [0254] This example describes an exemplary embodiment of the present invention for the determination of different anti-drug antibody (ADA) isotypes such as different isotypes of ATI (*i.e.*, antibody to IFX; “HACA”) in ADA-positive patients receiving anti-TNF α

drug therapy such as infliximab (IFX). Non-limiting examples of antibody isotypes such as ATI isotypes include IgA, IgD, IgE, IgG, and IgM (*e.g.*, IgA ATI, IgD ATI, IgE ATI, IgG ATI, and IgM ATI). In certain aspects, the detection of the presence or level of a specific ADA isotype or a particular combination of ADA isotypes is associated with
5 different clinical outcomes.

[0255] Figures 30 and 31 illustrate embodiments of the proximity-based isotyping assay of the present invention for determining the presence (or absence) or level of at least one, two, three, four, five, or more ATI isotypes such as, *e.g.*, IgA ATI, IgD ATI, IgE ATI, IgG ATI, and/or IgM ATI isotypes using fluorescence resonance energy transfer (FRET). For
10 example, Figure 30 shows a schematic illustrating that Alexa-532 (“F2” label on an anti-Ig antibody such as anti-IgA) is excited by Alexa-488 (“F1” label on antibody therapeutic such as IFX) only when both fluorophores are in close proximity, thereby indicating the presence and/or level of a ternary complex of an anti-Ig (*e.g.*, anti-IgA), HACA (*e.g.*, IgA ATI), and IFX. In particular embodiments, different ATI isotypes and/or subclasses
15 thereof can be determined using different anti-Ig’s labeled with the same or different fluorophores such as, *e.g.*, Alexa-532. Figure 31 shows the results of a FRET-based isotyping assay of the present invention.

[0256] 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
20 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 at least one isotype of an autoantibody to an anti-TNF α drug in a sample, the method comprising:
 - (a) contacting a labeled anti-TNF α drug with a sample having or suspected of having at least one isotype of an autoantibody to the anti-TNF α drug to form labeled complexes between the labeled anti-TNF α drug and each autoantibody isotype;
 - (b) subjecting the labeled complexes to size exclusion chromatography to separate the labeled complexes having different autoantibody isotypes from each other and from free labeled anti-TNF α drug; and
 - (c) detecting the labeled complexes, thereby detecting the presence or level of at least one isotype of the autoantibody to the anti-TNF α drug.
2. The method of claim 1, wherein the anti-TNF α drug is selected from the group consisting of REMICADETM (infliximab), HUMIRATM (adalimumab), ENBRELTM (etanercept), CIMZIA[®] (certolizumab pegol), and combinations thereof.
3. The method of claim 1 or 2, wherein the autoantibody to the anti-TNF α 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 at least one isotype comprises a plurality of at least two, three, four, five, or more isotypes.
5. The method of any one of claims 1 to 4, wherein the at least one isotype is selected from the group consisting of IgA, IgD, IgE, IgG, and IgM isotypes, subclasses thereof, and combinations thereof.
6. The method of any one of claims 1 to 5, wherein the sample is whole blood, serum, or plasma.
7. The method of any one of claims 1 to 6, wherein the autoantibody to the anti-TNF α drug is HACA and the sample is obtained from a subject on REMICADETM (infliximab) therapy.

8. The method of any one of claims 1 to 6, wherein the autoantibody to the anti-TNF α drug is HAHA and the sample is obtained from a subject on HUMIRATM (adalimumab) therapy.
9. The method of any one of claims 1 to 8, wherein the labeled anti-TNF α drug is a fluorophore-labeled anti-TNF α drug.
10. The method of any one of claims 1 to 9, wherein the labeled complexes are detected using fluorescence label detection.
11. The method of any one of claims 1 to 10, wherein each autoantibody isotype is characterized or identified by its retention time.
12. The method of any one of claims 1 to 11, wherein the size exclusion chromatography is size exclusion-high performance liquid chromatography (SE-HPLC).
13. A method for detecting the presence or level of at least one isotype of an autoantibody to an anti-TNF α drug in a sample, the method comprising:
- (a) contacting a labeled anti-TNF α drug and one or a plurality of labeled anti-Ig antibodies specific for different antibody isotypes with a sample having or suspected of having at least one isotype of an autoantibody to the anti-TNF α drug to form labeled complexes between the labeled anti-TNF α drug, the labeled anti-Ig antibodies, and each autoantibody isotype, wherein the labeled anti-TNF α drug and the labeled anti-Ig antibodies comprise different labels;
 - (b) subjecting the labeled complexes to size exclusion chromatography to separate the labeled complexes having different autoantibody isotypes from each other, from free labeled anti-TNF α drug, and/or from free labeled anti-Ig antibodies; and
 - (c) detecting the labeled complexes, thereby detecting the presence or level of at least one isotype of the autoantibody to the anti-TNF α drug.
14. The method of claim 13, wherein the anti-TNF α drug is selected from the group consisting of REMICADETM (infliximab), HUMIRATM (adalimumab), ENBRELTM (etanercept), CIMZIA[®] (certolizumab pegol), and combinations thereof.

15. The method of claim 13 or 14, wherein the autoantibody to the anti-TNF α 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.
- 5 16. The method of any one of claims 13 to 15, wherein the labeled anti-TNF α drug and the one or plurality of labeled anti-Ig antibodies bind to different epitopes of the at least one isotype.
17. The method of any one of claims 13 to 16, wherein the at least one isotype comprises a plurality of at least two, three, four, five, or more isotypes.
- 10 18. The method of any one of claims 13 to 17, wherein the at least one isotype is selected from the group consisting of IgA, IgD, IgE, IgG, and IgM isotypes, subclasses thereof, and combinations thereof.
- 15 19. The method of any one of claims 13 to 18, wherein the plurality of labeled anti-Ig antibodies comprises at least two, three, four, five, or more labeled anti-Ig antibodies specific for different antibody isotypes.
- 20 20. The method of any one of claims 13 to 19, wherein the one or plurality of labeled anti-Ig antibodies is selected from the group consisting of antibodies specific for one or more of IgA, IgD, IgE, IgG, and IgM isotypes, subclasses thereof, and combinations thereof.
21. The method of any one of claims 13 to 20, wherein the sample is whole blood, serum, or plasma.
22. The method of any one of claims 13 to 21, wherein the autoantibody to the anti-TNF α drug is HACA and the sample is obtained from a subject on REMICADETM (infliximab) therapy.
- 25 23. The method of any one of claims 13 to 21, wherein the autoantibody to the anti-TNF α drug is HAHA and the sample is obtained from a subject on HUMIRATM (adalimumab) therapy.

24. The method of any one of claims 13 to 23, wherein the labeled anti-TNF α drug is a fluorophore-labeled anti-TNF α drug.

25. The method of any one of claims 13 to 24, wherein the labeled anti-Ig antibodies are fluorophore-labeled anti-Ig antibodies.

5 26. The method of any one of claims 13 to 25, wherein the plurality of labeled anti-Ig antibodies specific for different antibody isotypes each comprise the same label or different labels.

27. The method of any one of claims 13 to 26, wherein the labeled complexes are detected using fluorescence label detection.

10 28. The method of any one of claims 13 to 27, wherein the labeled complexes are detected upon a signal that is generated by the proximity binding of both the labeled anti-TNF α drug and the labeled anti-Ig antibodies to the autoantibody isotype.

29. The method of claim 28, wherein the signal comprises a fluorescent signal that is detected by fluorescence resonance energy transfer (FRET).

15 30. The method of any one of claims 13 to 29, wherein the size exclusion chromatography is size exclusion-high performance liquid chromatography (SE-HPLC).

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