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(54) **Title:** BIOMARKERS FOR CHAGAS DISEASE RELATED CARDIOMYOPATHY

(57) **Abstract:** Certain embodiments include methods for assessing a subject having a trypanosome infection for the presence or absence of indications of cardiomyopathy.



BIOMARKERS FOR CHAGAS DISEASE RELATED CARDIOMYOPATHY

## STATEMENT REGARDING PRIORITY

[001] This Application claims priority to U.S. Provisional Patent Application No. 61/775,676 filed March 10, 2013, which is incorporated herein by reference in its entirety. This Application claims priority to and is a continuation-in-part of U.S. Patent Application No. 13/470,209 filed May 11, 2012, which claims priority to U.S. Provisional Patent Application No. 61/518,736 filed May 11, 2011, each of which is incorporated herein by reference in their entirety.

## STATEMENT REGARDING FEDERALLY FUNDED RESEARCH

[002] This invention was made with government support under HHSN272200800048C and HL094802 awarded by the National Institutes of Health. The United States government has certain rights in the invention.

## BACKGROUND

[003] Chagas disease, transmitted by injection of *Trypanosoma cruzi* through the bite of an insect vector, is designated as the most important emerging disease in developed countries, with approximately 16-18 million cases of infection in Latin America with 120 million people (~25% of the population) more at risk of infection (Organization, W. H. (2010) Chagas disease: control and elimination, In *Report of the secretariat. WHO, Geneva, UNDP/World Bank/WHO*). In 30-40% of the infected individuals, the disease may progress to irreversible cardiomyopathy after many years, with infected individuals serving as carriers of the organism and exhibiting considerable morbidity and high risk of mortality (Machado et al. (2012) *Front Biosci (Elite Ed)* 4, 1743-58). Unfortunately, there are no vaccines or safe drugs - benznidazole and nifurtimox can be used for treatment of acute infection, but have high toxicity in adults and are ineffective in arresting or reversing the progression of the disease—and as a consequence, the NIH and CDC have recognized Chagas disease as a neglected emergency (Bern and Montgomery, (2009) *Clin Infect Dis* 49, e52-54; CDC. (2006) Chagas disease after organ transplantation--Los Angeles, California, 2006, *MMWR Morb Mortal Wkly Rep* 55, 798-800; CDC. (2007) Blood donor screening for chagas disease--United States, 2006-2007, *MMWR Morb Mortal Wkly Rep* 56, 141-143).

[004] Thus, there remains a need for additional compositions and methods for identifying subjects harboring Trypanosomes and particularly those subjects at risk of developing cardiomyopathy.

## SUMMARY

[005] Blood serves as a useful tissue capable of detecting and responding to the changes induced in the body during the course of *T. cruzi* infection and disease development. The changes in immune response, oxidative stress, and antioxidant imbalance are detectable in peripheral blood of infected mice (Wen et al., (2008) *Microbes Infect*, 10, 1201-09), and, notably, a strong positive correlation was detected for the disease state-specific changes in the heart-versus-blood level of oxidative stress markers and antioxidants (e.g. glutathione peroxidase, glutathione, manganese superoxide dismutase) (Wen et al., (2008) *Microbes Infect*, 10, 1201-09). Distinct plasma protein-nitrosylation profiles have also been documented in acutely- and chronically-infected chagasic animals (Dhiman et al., (2008) *Am J Pathol*, 173, 728-740). Studies described herein along with documentation of oxidative overload in chagasic humans (Wen et al., (2006) *Free Rad Biol Med*, 41, 270-76; de Oliveira et al., (2007) *Int J Cardiol*, 116, 357-63), support the idea that characterization of plasma proteomes will be useful in identifying the molecular mechanisms that are disturbed during the progression of Chagas disease.

[006] Certain embodiments include assessing the status of a subject by measuring and evaluating the protein levels and/or the levels of protein modification of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more proteins selected from vinculin (SEQ ID NO:1), serum albumin (SEQ ID NO:2), integrin alpha-IIb isoform 3 (SEQ ID NO:3), myeloperoxidase isoform H7 (SEQ ID NO:4), actin (cytoplasmic 2, N-terminal processed) (SEQ ID NO:5), Talin 1 (SEQ ID NO:6), actin (cytoplasmic 1, N-terminal processed) (SEQ ID NO:7), actin (cytoplasmic 1, N-terminal processed) (SEQ ID NO:8), unconventional myosin-IXa (SEQ ID NO:9), peptidyl-prolyl cis-trans isomerase A (SEQ ID NO:10), WD repeat-containing protein 49 (SEQ ID NO:11), Keratin type II cytoskeletal 1 (SEQ ID NO:12), parathyroid hormone 2 receptor (fragment) (SEQ ID NO:13), proteasome subunit beta type-2 (SEQ ID NO:14), ferritin light chain (SEQ ID NO:15), annexin (SEQ ID NO:16), actin (cytoplasmic 1, N-terminal processed) (SEQ ID NO:16), keratin type I

cytoskeletal 10 (SEQ ID NO:18), heterogeneous nuclear ribonucleoprotein A1 (Fragment) (SEQ ID NO:19), SH3 domain-binding glutamic acid-rich-like protein 3 (SEQ ID NO:20), Ras-related protein Rap-1b (SEQ ID NO:21), actin (cytoplasmic 1, N-terminal processed) (SEQ ID NO:22), POTE ankyrin domain family member F (SEQ ID NO:23), vimentin (SEQ ID NO:24), protein S100-A11 (SEQ ID NO:25), Isoform 2 of fibrinogen alpha chain (SEQ ID NO:26), tubulin beta chain (SEQ ID NO:27), Myosin regulatory light chain 12B (SEQ ID NO:28), Annexin A3 (SEQ ID NO:29), keratin type I cytoskeletal 10 (SEQ ID NO:30), Actin cytoplasmic 2 N-terminally processed (Fragment) (SEQ ID NO:31), ATP synthase subunit alpha (SEQ ID NO:32) (see table III). The methods further comprising computer implementation of such a method. In certain aspects the subject is diagnosed or at risk of trypanosome infection. In further aspects the subject has a trypanosome infection and is assessed for the presence or absence of indications of cardiomyopathy.

[007] Certain embodiments include methods of assessing a subject having Chagas disease comprising: measuring levels of (i) one or more proteins selected from vimentin, gamma actin, or keratin 10, or (ii) level of cysteinyl-S-nitrosylation (SNO) of one or more of beta actin, annexin A6, ferritin light chain fragment (~18kDa), parathyroid hormone 2 receptor fragment (~20kDa), or myosin-IXa fragment; wherein elevated levels of the proteins and/or nitrosylation is indicative of risk for developing chagasic cardiomyopathy. Certain protein levels will increase and certain protein levels will decrease as compared to control. SNO modification will either increase or decrease relative to a control. The levels of the markers have been associated with a particular disease state. As SNO modification increases the fluorescence from the modified protein decreases. Likewise, as SNO modification decreases the fluorescence of the lesser-modified protein increases. In certain aspects a SNO modification is associated with cardiomyopathy as is indicated by a negative ratio in the CCM+ group and a positive ratio in the CCM- group. In a further aspect a SNO modification is associated with a non-cardiomyopathic condition in which the CCM- ratio is negative and the CCM+ ratio is positive. Furthermore, an increased or decreased level of a protein can be associated with CCM+ as is indicated by a positive or negative abundance ratio, respectively.

[008] In certain aspects the level of vimentin is measured. In a further aspect the level of gamma actin is measured. In certain aspects the level of keratin 10 is measured. In certain aspects the levels of vimentin and gamma actin are measured. In certain aspects the level of

vimentin and keratin 10 are measured. In certain aspects the level of gamma actin and keratin 10 are measured. In certain aspects the level of vimentin, gamma actin, and keratin 10 are measured. In certain aspects protein levels are determined by western blot analysis, mass spectrometry, or image analysis of two-dimensional gels.

[009] In certain aspects the level of modification of ferritin light chain fragment is measured. In certain aspects the level of modification of annexin A6 is measured. In certain aspects the level of modification of myosin-IXa fragment is measured. In certain aspects the level of modification of ferritin light chain fragment and annexin A6 is measured. In certain aspects the level of modification of ferritin light chain fragment and myosin-IXa fragment is measured. In certain aspects the level of modification of annexin A6 and myosin-IXa fragment is measured. In certain aspects the level of modification of ferritin light chain fragment, annexin A6, and myosin-IXa fragment is measured. In certain aspects the modification level of parathyroid hormone receptor and/or  $\beta$  actin are measured. In certain aspects the level of protein modification is measured by saturation fluorescence labeling.

[010] In certain aspects the protein level and/or SNO modification of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 of the proteins described Table III are measured.

[011] Certain aspects include a computer-implemented method for assessing a subject for trypanosome infection or cardiomyopathy. In certain aspects a computer implemented method comprises the steps of (a) obtaining protein level measurements of one or more of vimentin, gamma actin, or keratin 10, and/or protein modification measurements of one or more of beta actin, annexin A6, ferritin light chain fragment (~18kDa), parathyroid hormone 2 receptor fragment (~20kDa), or myosin-IXa fragment, (b) transforming the obtained measurements to a score or ratio, and (c) determining if the measurements indicate the presence of trypanosome infection or the risk of developing or the presence or absence of cardiomyopathy.

[012] Certain aspects include methods of treating a patient at risk of or having initial indication of chagasic cardiomyopathy comprising: administering a treatment for cardiomyopathy to a patient having elevated levels of (i) one or more proteins selected from vimentin, gamma actin, or keratin 10, or (ii); level of cysteinyl-S-nitrosylation (SNO) of one

or more of beta actin, annexin A6, ferritin light chain fragment (~18kDa), parathyroid hormone 2 receptor fragment (~20kDa), or myosin-IXa fragment

[013] The treatment of cardiomyopathy includes administration of vasodilators (e.g., prazosin, hydralazine and the like), angiotensin conversion enzyme inhibitors (e.g., captopril and the like) and the like for symptomatic therapy of dilated cardiomyopathy; and administration of  $\beta$  blockers (e.g., propranolol and the like) and Ca antagonists (e.g., verapamil, diltiazem and the like) to treat hypertrophic cardiomyopathy.

[014] Certain embodiments include detecting evidence of chagasic cardiomyopathy in a biological sample, comprising the step of measuring the level or presence of at least one protein selected from the group consisting of gelsolin (GSN), myosin light chain 2 (MYL2), vimentin (VIM), myosin heavy chain 11 (MYH11), vinculin (VCL), and plasminogen (PLG) in the sample. In an undiagnosed subject, the levels of one or more of the proteins can be indicative of *T. cruzi* infection. In a subject already diagnosed with *T. cruzi* infection, the protein levels can be indicative of the severity of disease, e.g., the risk of developing, or the stage of chagasic cardiomyopathy in the subject. Elevated levels of gelsolin (GSN), myosin light chain 2 (MYL2), vimentin (VIM), myosin heavy chain 11 (MYH11), vinculin (VCL), and/or plasminogen (PLG) biomarkers in the samples is indicative of *T. cruzi* infection, Chagas disease, and/or chagasic cardiomyopathy in the subject. The content of US Application serial number 13/470,209 filed May 11, 2012 entitled "Diagnostic Methods for Assessing Risk of Chagas Disease and Heart Failure" is incorporated herein by reference in its entirety.

[015] Certain aspects may include assessment of a subject for risk of developing chagasic cardiomyopathy. Levels of biomarkers are measured and these measurements indicate whether a subject is at risk of developing cardiomyopathy. Certain aspects include measuring the levels of VIM, GSN, MYL2, MYH11, VCL, and PLG. In a further aspect, levels of VIM are measured in combination with one or more of GSN, MYL2, MYH11, VCL, or PLG. In a further aspect, levels of GSN are measured in combination with one or more of VIM, MYL2, MYH11, VCL, or PLG. In still a further aspect, levels of MYL2 are measured in combination with one or more of VIM, GSN, MYH11, VCL, or PLG. Certain aspects include measuring levels of MYH11 in combination with one or more of VIM, GSN,

MYL2, VCL, or PLG. In further aspects, levels of VCL are measured in combination with one or more of VIM, GSN, MYL2, MYH11, or PLG. In still a further aspect, levels of PLG are measured in combination with one or more of VIM, GSN, MYL2, MYH11, or VCL.

[016] In certain aspects, the methods include treating a subject identified as (a) having *T. cruzi* infection, (b) at risk of developing chagasic cardiomyopathy, or (c) diagnosed with chagasic cardiomyopathy. Treatments can include anti-trypanosome treatments, or preventive or therapeutic treatments for cardiomyopathy, or a combination of both.

[017] Certain aspects include methods for screening blood comprising measuring the levels of one or more of VIM, GSN, MYL2, MYH11, VCL, and PLG proteins. An increased level of one or more of these proteins is indicative of *Trypanosome* contamination. In certain aspects blood is screened prior to or during banking. In a further aspect, the methods can further comprise conducting confirmatory testing if the levels of one or more of the biomarkers are elevated.

[018] Certain embodiments are directed to serodiagnostic kits for determining whether a subject is infected with *Trypanosoma cruzi* and/or staging the severity of Chagas disease, said kit comprising: (a) an antibody directed against VIM, GSN, MYL2, MYH11, VCL, and/or PLG, wherein said antibody is linked to a reporter molecule; (b) a buffer; and c) a reagent for detection of the reporter molecule.

[019] Other embodiments of the invention are discussed throughout this application. Any embodiment discussed with respect to one aspect of the invention applies to other aspects of the invention as well and vice versa. Each embodiment described herein is understood to be embodiments of the invention that are applicable to all aspects of the invention. It is contemplated that any embodiment discussed herein can be implemented with respect to any method or composition of the invention, and vice versa. Furthermore, compositions and kits of the invention can be used to achieve methods of the invention.

[020] The term “antigen” as used herein is defined as a compound, composition, or substance that can stimulate the production of antibodies or a T cell response in an animal, including compositions that are injected or absorbed into an animal. An antigen reacts with the products of specific humoral or cellular immunity, including those induced by

heterologous immunogens. The term “antigen” includes all related antigenic epitopes. “Epitope” or “antigenic determinant” refers to a site on an antigen to which B and/or T cells respond. Epitopes can be formed both from contiguous amino acids or noncontiguous amino acids juxtaposed by tertiary folding of a protein.

[021] The term “antibody” as used herein includes immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antigen binding site that specifically binds (immunoreacts with) an antigen. A naturally occurring antibody (e.g., IgG, IgM, IgD) includes four polypeptide chains, two heavy (H) chains and two light (L) chains interconnected by disulfide bonds. However, it has been shown that the antigen-binding function of an antibody can be performed by fragments of a naturally occurring antibody. Specific, non-limiting examples of binding fragments encompassed within the term antibody include (i) a Fab fragment consisting of the  $V_L$ ,  $V_H$ ,  $C_L$  and  $C_{H1}$  domains; (ii) an  $F_d$  fragment consisting of the  $V_H$  and  $C_{H1}$  domains; (iii) an Fv fragment consisting of the  $V_L$  and  $V_H$  domains of a single arm of an antibody, (iv) a dAb fragment (Ward et al., Nature 341:544-546, 1989); and (vi) a  $F(ab')_2$  fragment. Immunoglobulins and certain variants thereof are known and many have been prepared in recombinant cell culture (e.g., see U.S. Pat. No. 4,745,055; U.S. Pat. No. 4,444,487; WO 88/03565; EP 256,654; EP 120,694; EP 125,023; Falkner et al., Nature 298:286, 1982; Morrison, J. Immunol. 123:793, 1979; Morrison et al., Ann Rev. Immunol 2:239, 1984).

[022] The term “animal” as used herein refers to living multi-cellular vertebrate organisms, a category that includes, for example, mammals and birds. The term mammal includes both human and non-human mammals. Similarly, the term “subject” includes both human and veterinary subjects. The term mammal includes dogs, cats, cattle, horses, goats, sheep, and other domesticated mammals, as well as non-domesticated mammals. In particular embodiments, the animal is a triatome (the insect vector of *T. cruzi*) host, e.g., a human, opossum, raccoon, armadillo, squirrel, rat, or mouse.

[023] The term “diagnostic” refers to identifying the presence or nature of a pathologic condition. Diagnostic methods differ in their sensitivity and specificity. The “sensitivity” of a diagnostic assay is the percentage of diseased individuals who test positive (percent of true positives). The “specificity” of a diagnostic assay is 1 minus the false positive rate, where

the false positive rate is defined as the proportion of those without the disease who test positive. While a particular diagnostic method may not provide a definitive diagnosis of a condition, it suffices if the method provides a positive indication that aids in diagnosis.

[024] “Prognosis” is a probability that a pathologic condition will develop (e.g., result in additional sequelae) or progress (e.g., increase in severity).

[025] The use of the word “a” or “an” when used in conjunction with the term “comprising” in the claims and/or the specification may mean “one,” but it is also consistent with the meaning of “one or more,” “at least one,” and “one or more than one.”

[026] Throughout this application, the term “about” is used to indicate that a value includes the standard deviation of error for the device or method being employed to determine the value.

[027] The use of the term “or” in the claims is used to mean “and/or” unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and “and/or.”

[028] As used in this specification and claim(s), the words “comprising” (and any form of comprising, such as “comprise” and “comprises”), “having” (and any form of having, such as “have” and “has”), “including” (and any form of including, such as “includes” and “include”) or “containing” (and any form of containing, such as “contains” and “contain”) are inclusive or open-ended and do not exclude additional, unrecited elements or method steps.

[029] Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating specific embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

## DESCRIPTION OF THE DRAWINGS

[030] The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of the specification embodiments presented herein.

[031] **FIG. 1.** Saturation fluorescence labeling with uncharged cysteine-specific BODIPY-FL-maleimide (BD) dye. Three cases are presented focusing on the degree of cysteine modification reversible by Asc (i.e., SNO). All assume that the protein abundance does not change with the treatment. Case A demonstrates a canonical protein structure with no SNO before treatment with BD, after labeling, and after Asc-treatment and labeling. No change in protein spot fluorescence will be observed unless the protein abundance changes. This is quantified by the ratios of ratios as described in the Methods. Case B demonstrates the partial SNO after treatment and the resultant ratio of ratios upon Asc-treatment and labeling. Here ratios less than 1 are expressed as the negative reciprocal. Case C demonstrates the complete SNO and the ratio of ratios after Asc-reversal. If the protein abundance changes due to the cardiomyopathy, with or without SNO, the ratio of ratios will normalize the values. Taken from ref [Wiktorowicz et al., (2011) *Biochemistry* 50, 5601-5614.].

[032] **FIG. 2.** Variable Importance for MARS model of CCM+. Variable importance was computed for each feature in the MARS model. Y-axis, percent contribution for each analyte.

[033] **FIG. 3.** ROC analysis. Shown is a Receiver Operating Characteristic (ROC) curve for the predictive model for CCM+. Y-axis, Sensitivity; X-axis, 1-Specificity.

[034] **FIG. 4.** 2DE images. Shown is a reference gel of 2DE of proteins from the chagasic study subjects. The locations and identities of protein spots that contribute to the prediction of chagasic cardiomyopathy are indicated. SNO = proteins whose SNO modification is discriminatory; AM = proteins whose abundance is discriminatory.

[035] **FIG. 5.** Box plot of spot 385 identified as an abundance modulated vimentin.

[036] **FIG. 6.** Box plot of spot 572 identified as an SNO modified ferritin light-chain.

- [037] **FIG. 7.** Box plot of spot 411 identified as an SNO modified myosin fragment.
- [038] **FIG. 8.** Box plot of spot 524 identified as an abundance modulated keratin 10.
- [039] **FIG. 9.** Box plot of spot 627 identified as an SNO modified annexin A6.
- [040] **FIG. 10.** Box plot of spot 563 identified as an abundance modulated gamma actin.
- [041] **FIG. 11.** Probability of inclusion parameters from Stochastic Search Variable Selection model.
- [042] **FIG. 12.** SSVS model differential 2DE spot abundance in chagasic cardiomyopathy. Shown is a box-plot comparison of 2DE spot values between CCM+ and CCM- for spot 506, SNO modified parathyroid hormone receptor.
- [043] **FIG. 13.** SSVS model box-plot comparison of 2DE spot values between CCM+ and CCM- for spot 758, SNO modified beta actin.
- [044] **FIG. 14.** Illustration of computer implementation.

#### DESCRIPTION

[045] In 30-40% of trypanosome infected individuals disease may progress to irreversible cardiomyopathy after many years with infected individuals serving as carriers of the organism and exhibiting considerable morbidity and high risk of mortality (Machado et al., (2012) *Front Biosci (Elite Ed)* 4, 1743-58). Unfortunately, there are no vaccines or safe drugs - benznidazole and nifurtimox can be used for treatment of acute infection, but have high toxicity in adults and are ineffective in arresting or reversing the progression of the disease. Accordingly, it is crucial that biomarkers and molecular pathways are identified that could classify the disease state, detect asymptomatic individuals who are at risk of developing chagasic cardiomyopathy, identify new therapies to arrest or prevent the progression of symptomatic clinical disease, and develop tools, methods and kits to assess the efficacy of new therapies.

[046] Distinct cardiac and plasma proteins have been identified as being oxidized/nitrated in acute and chronic chagasic animals and humans (Dhiman et al., (2008)

*Am J Pathol* 173, 728-40; Wen and Garg, (2012) *Mol Cell Proteomics* 11, M111.010918. Epub 012011; Wen et al., (2012) *Mol Cell Proteomics* 11, 435-52), and there is a direct correlation between cardiac and peripheral blood level of protein oxidation in chagasic mice (Wen et al., (2008) *Microbes Infect* 10, 1201-09). These observations suggest that pathological processes leading to the development of chagasic cardiomyopathy in patients cause characteristic changes in the concentration/oxidation of proteins in the blood and generate a detectable disease-specific molecular phenotype. The inventors have identified combinations of proteins and oxidatively modified proteins (cysteiny-S-nitrosylated, SNO) that can be used to assess the risk of developing irreversible cardiomyopathy. Certain embodiments include one or more of:

[047] Identifying and/or using a set of proteins whose concentration and/or SNO modifications can be measured in a patient with or without diagnosed Chagas disease to indicate the presence of trypanosome infection and/or cardiomyopathy. The predictive proteins can include, but are not limited to vimentin, gamma actin, and keratin 10. The predictive SNO proteins include, but are not limited to beta actin, annexin A6, ferritin light chain fragment (~18kDa), parathyroid hormone 2 receptor fragment (~20kDa), and a myosin-IXa fragment.

[048] Identifying and/or using a set of proteins whose concentration or SNO modifications can be measured in a patient with Chagas cardiomyopathy that would indicate progression of cardiomyopathy, or the patient's response to treatment.

[049] Formulating a predictive model consisting of weighted combinations of the same measured levels of specific proteins and SNO modified proteins that indicate risk for developing cardiomyopathy.

[050] *Clinical human cohorts.* The initial phase of one study utilized representative PBMC samples from seropositive chagasic patients that were clinically characterized in Argentina according to published protocols (Wen et al., (2012) *Mol Cell Proteomics* 11, 435-52). In the state of Salta, at the border of Argentina-Bolivia, Chagas disease is endemic. Patients were tested to be seropositive for *T. cruzi* infection, characterized for cardiac function, and grouped as:

[051] *CCM- group.* The CCM- group included 25 seropositive, asymptomatic subjects with no indication of cardiac involvement (i.e., no echocardiography abnormalities, preserved systolic function (ejection fraction (EF)  $\geq 55\%$ ), no left ventricular dilatations, and negligible-to-minor EKG alterations).

[052] *CCM+ group.* The CCM+ group included 28 seropositive, symptomatic chagasic patients with a degree of systolic dysfunction (EF:  $\leq 40-54\%$ ) and/or left ventricular dilatation (diastolic diameter  $\geq 57$  mm).

[053] Classification of Chagas disease was done according to the NYHA Guidelines.

[054] The inventors employ a rigorous blood collection and storage protocol to ensure sample quality. Briefly, heparinized blood (10 ml/subject) is centrifuged at 1000 g, 4°C for 20 min and the resulting buffy-coat transferred to a new tube containing percoll (1.084 g/L) cushion. After centrifugation as above, PBMCs lying at the PBS-percoll interface are transferred to a new tube, and washed with cold PBS. PBMC pellets are stored at -80°C for future use.

[055] *Saturation Fluorescence Labeling and Cysteinyl-S-Nitrosylation by Fluorescence (SNOFlo).* After cysteine (cysteic acid) content was determined by amino acid analysis (Model L8800, Hitachi High Technologies America, Pleasanton, CA), the proteins obtained from PBMCs of Chagasic patients (+/- cardiomyopathy) by urea/thiourea extraction were split into two equal pools. One pool was processed for quantitative saturation fluorescence labeling with uncharged BODIPY FL-maleimide (BD) at a dye-to-protein thiol ratio of greater than 50:1 ratio. This saturation fluorescence labeling method has yielded high accuracy (>91%) in quantifying blinded protein samples (Pretzer and Wiktorowicz, (2008) *Anal Biochem* 374, 250-262).

[056] The second pool was first treated with 6 mM ascorbic acid (Asc) to remove S-nitrosyl (SNO) groups from cysteine residues. These samples were then dialyzed against the urea/thiourea extraction buffer, after which the proteins were labeled with BD as above.

[057] The BD-labeled proteins were separated using two-dimensional gel electrophoresis (2DE) employing an IPGphor multiple sample IEF device (Pharmacia,

Piscataway, NJ) in the first dimension, and Protean Plus and Criterion Dodeca cells (Bio-Rad, Hercules, CA) in the second dimension (Jamaluddin et al., (2010) *J Virol* 84, 9533-45). Sample aliquots were first loaded onto 11 cm dehydrated precast immobilized pH gradient (IPG) strips (Bio-Rad), and rehydrated overnight. IEF was performed at 20°C with the following parameters: 50 Volts, 11 h; 250 V, 1 h; 500 V, 1 h; 1000 V, 1 h; 8000 V, 2 h; 8000 V, 6 h. The IPG strips were then be incubated in 4 mL of equilibration buffer (6 M urea, 2% SDS, 50 mM Tris-HCl, pH 8.8, 20% glycerol) containing 10 µl/mL tri-2 (2-carboxyethyl) phosphine (Geno Technology, Inc., St. Louis, MO) for 15 minutes at 22°C with shaking. The samples were incubated in another 4 mL of equilibration Buffer with 25 mg/mL iodoacetamide for 15 min at 22°C with shaking in order to ensure protein S-alkylation of any residual reduced thiol groups. Electrophoresis was performed at 150 V for 2.25 h, 4°C with precast 8-16% polyacrylamide gels in Tris-glycine buffer (25 mM Tris-HCl, 192 mM glycine, 0.1% SDS, pH 8.3) ( Pretzer and Wiktorowicz, (2008) *Anal Biochem* 374, 250-262).

[058] *Protein Fluorescence Staining.* After electrophoresis, the gels were directly imaged at 100 µm resolution using the GE Healthcare Typhoon Trio Proteomic Imaging System to quantify BD-labeled proteins (>90% of human proteins contain at least one cysteine (Miseta and Csutora, (2000) *Mol Biol Evol* 17, 1232-39). A gel containing the most common features was selected by Nonlinear Samespots software (see below) as the reference gel for the entire set of gels. The gel was destained in 20% ethanol/10% acetonitrile, washed with water, and scanned at 485/550 nm (ex/em). The exposure time was adjusted to achieve a value of ~55,000-63,000 pixel intensity (16-bit saturation) from the most intense protein spots on the gel.

[059] *Measurement of relative spot intensities.* The 2D gel images were analyzed using Progenesis/SameSpots software (Nonlinear Dynamics, Ltd. Newcastle Upon Tyne, UK). The reference gel was selected according to quality and number of spots. Spots boundaries were established after automated “pixel to pixel” matching with manual adjustment, boundaries were examined to ensure proper distinction, and the gel images were used to obtain the quantitative spot data. This strategy ensures that spot numbers and outlines were identical across all gels in the experiment, eliminating problems with unmatched spots (Dowsey et al., (2010) *Methods Mol Biol* 604, 239-55;13, 14) as well as ensuring that the greatest number of protein spots and their spot volumes were accurately detected and quantified. Spot volumes

were normalized using a software-calculated bias value assuming that the great majority of spot volumes did not change in abundance.

[060] *SNOFlo Data Processing.* SNOFlo uses a thiol-reactive fluorescent label to detect free thiols in proteins. The presence of a nitrosyl (NO) group will prevent the labeling of thiols, so that when compared to the same sample for which all NO groups have been removed (Asc+ treatment), SNO labeled proteins will yield spot volumes less than the Asc+ treatment (no NO modification; FIG. 1)( Wiktorowicz et al., (2011) *Biochemistry* 50, 5601-14). However, since one goal was to establish classifiers of chagasic cardiomyopathy, variation in protein intensities (spot volumes) may reflect changes in SNO status, changes in protein abundance, or both. Treatment of half of a patient PBMC extract with Asc removes all NOs, and spot volume ratio within Asc+ treatment, but across patient cardiac status, yields an estimate of the change in protein abundance, while spot volume ratios within Asc-treatment yields a combined estimate of change in SNO and/or protein abundance. Thus in order to normalize the SNO from abundance changes, the inventors calculate a ratio of ratios of Asc- ratios with Asc+ treatment ratios of each protein spot and a normalized SNO ratio according to the equations as follows:

$$\text{Ratio of ratios} = \frac{[BD_{\text{Asc}^-}^{\text{Exp}} / BD_{\text{Asc}^-}^{\text{Ctrl}}]}{[BD_{\text{Asc}^+}^{\text{Exp}} / BD_{\text{Asc}^+}^{\text{Ctrl}}]} = \frac{\Delta [\text{Cys-NO}]}{\Delta [\text{protein}]}$$

where BD = normalized BODIPY fluorescence intensity of a protein spot, Asc<sup>-</sup> = non-Asc treated, Asc<sup>+</sup> = Asc treated, Exp = CCM+, Ctrl = CCM-.

[061] However, since particular statistical model building software requires single values from each patient within his/her cohort, only the Asc-/Asc+, and Asc+ ratios were used to determine classifiers, as follows:

$$\text{Normalized SNO Ratio} = \frac{[BD_{\text{Asc}^-}]}{[BD_{\text{Asc}^+}]} = \frac{[\text{Cys-NO}]}{[\text{protein}]}$$

to reflect the SNO status of each protein spot, or:

$$\text{Protein Abundance Ratio} = \frac{[BD_{\text{Asc}^+}^{\text{Exp}}]}{[BD_{\text{Asc}^+}^{\text{Ctrl}}]} = \Delta [\text{protein}]$$

to reflect the difference in protein abundance CCM+ to CCM-.

[062] *Protein Identification.* Selected 2DE spots were picked robotically, trypsin-digested, and peptide masses identified by MALDI TOF/TOF (AB Sciex 5800, Foster City, CA). Data were analyzed with the Applied Biosystems software package included 4000 Series Explorer (v. 3.6 RC1) with Oracle Database Schema Version (v. 3.19.0), Data Version (3.80.0) to acquire both MS and MS/MS spectral data. The instrument was operated in positive ion reflectron mode, mass range was 850 – 3000 Da, and the focus mass was set at 1700 Da. For MS data, 2000-4000 laser shots were acquired and averaged from each sample spot. Automatic external calibration was performed using a peptide mixture with reference masses 904.468, 1296.685, 1570.677, and 2465.199.

[063] Following MALDI MS analysis, MALDI MS/MS was performed on several (5-10) abundant ions from each sample spot. A 1kV positive ion MS/MS method was used to acquire data under post-source decay (PSD) conditions. The instrument precursor selection window was +/- 3 Da. For MS/MS data, 2000 laser shots were acquired and averaged from each sample spot. Automatic external calibration was performed using reference fragment masses 175.120, 480.257, 684.347, 1056.475, and 1441.635 (from precursor mass 1570.700).

[064] Applied Biosystems GPS Explorer™ (v. 3.6) software was used in conjunction with MASCOT to search the respective protein database using both MS and MS/MS spectral data for protein identification. Protein match probabilities were determined using expectation values and/or MASCOT protein scores. MS peak filtering included the following parameters: mass range 800 Da to 4000 Da, minimum S/N filter = 10, mass exclusion list tolerance = 0.5 Da, and mass exclusion list (for some trypsin and keratin-containing compounds) included masses 842.51, 870.45, 1045.56, 1179.60, 1277.71, 1475.79, and 2211.1. For MS/MS peak filtering, the minimum S/N filter = 10 (Wen and Garg, (2012) *Mol Cell Proteomics* 11, M111.010918. Epub 012011; Wen et al., (2012) *Mol Cell Proteomics* 11, 435-52).

[065] For protein identification, the *Homo sapiens* taxonomy was searched in the NCBI database. Other parameters included the following: selecting the enzyme as trypsin; maximum missed cleavages = 1; fixed modifications included carbamidomethyl (C) for 2-D gel analyses only; variable modifications included oxidation (M); precursor tolerance was set

at 0.2 Da; MS/MS fragment tolerance was set at 0.3 Da; mass = monoisotopic; and peptide charges were only considered as +1 ((Wen and Garg, (2012) *Mol Cell Proteomics* 11, M111.010918. Epub 012011; Wen et al., (2012) *Mol Cell Proteomics* 11, 435-52).

[066] Protein identification was performed using a Bayesian algorithm (Zhang and Chait, (2000) *Anal Chem* 72, 2482-89) where matches were indicated by expectation score, an estimate of the number of matches that would be expected in that database if the matches were completely random. In some circumstances, confirmation of the protein identification was performed by LC-MS/MS (Orbitrap Velos, ThermoFinnegan, San Jose, CA).

[067] *Statistical analysis.* Statistical comparisons were performed using SPSSv18 (SPSS, Inc., Chicago, IL) and R.

[068] Multivariate Adaptive Regression Splines (MARS). Log base 2-transformed differential protein expression data and differential protein SNO data were used for MARS modeling. The MARS model specified 6 possible basis functions. MARS is a non-parametric regression method that uses piecewise linear spline functions (basis functions) as predictors. The basis functions are combinations of independent variables and so this method allows detection of feature interactions and performs well with complex data structures (Friedman and Roosen, (1995) *Stat Methods Med Res* 4, 197-217). MARS uses a two-stage process for constructing the optimal classification model. The first half of the process involves addition of basis functions until a user-specified number of basis functions have been reached. In the second stage, MARS deletes basis functions in order, starting with the basis function that contributes the least to the model until an optimum model is reached. Ten-fold generalized cross-validation was used to avoid over-fitting the classification model (Salford Systems, Inc).

[069] Stochastic Search Variable Selection (SSVS). Stochastic variable search is a procedure that selects promising subsets of predictor variables in the defined design matrix, which is based on embedding the entire regression setup in a hierarchical Bayes normal mixture model, where latent variables are used to specify choices of subsets (George and McCulloch, (1993) *J. American Statistical Association* 88, 881-89). Those subsets with higher probability can be identified by their more frequent appearance in the Gibbs sample. The inventors explored different methods to improve the classification accuracy, having

fewer misclassifications while using fewer predictors. The WinBUGS software for Bayesian analysis using Markov Chain Monte Carlo (MCMC) method was used.

[070] *Proteomic Analyses.* In one study the chagasic cohort consisted of 53 patients in total (25 CCM-; 28 CCM+) in which each sample was split in two with one treated with ascorbate (to reverse SNO modifications) and the other processed without ascorbate. After labeling and spot fluorescence quantification, the first treatment reflects the protein concentration, while the second reflects the degree of SNO modification. Both are useful for establishing candidate classifiers for CCM.

[071] Thus 106 2D gels were analyzed, resulting in the detection of 635 protein spots after filtering and manual examination of the gel images. Two separate ratios of each treatment class with respect to the comparison of CCM+ to CCM- patients were calculated as indicated above. Submitted for statistical analyses were the abundance ratio of each spot (Asc+) CCM+ : CCM-, and the normalized SNO ratio (Asc-/Asc+) of CCM+ separately from CCM-.

[072] *Statistical Analyses.* Multivariate Adaptive Regression Splines (MARS) is a nonparametric, multivariate regression method that can estimate complex nonlinear relationships by a series of spline functions of the predictor variables. Regression splines seek to find thresholds and breaks in relationships between variables and are very well suited for identifying changes in the behavior of individuals or processes over time. As a nonparametric approach, MARS does not make any underlying assumptions about the distribution of the predictor variables of interest. This characteristic is useful in chagasic cardiomyopathy modeling because protein expression values are not normally distributed, as would be required for the application of classical modeling techniques such as logistic regression. The basic concept behind spline models is to model using potentially discrete linear or nonlinear functions of any analyte over differing intervals. The resulting piecewise curve, referred to as a spline, is represented by basis functions within our model. Other studies have shown that MARS is a superior method in the prediction of nonparametric datasets to phenotypes (Brasier et al.,(2010) *Clin Transl Sci* 3, 147-57). To reduce over-fitting, the inventors restricted their analysis to models that incorporated one or fewer interaction terms.

[073] The MARS model specified 15 possible basis functions and allowed only 1 interaction term. Ten-fold cross-validation, a more accurate way to measure how efficiently the classifier has learned a concept base using the training set, was used to avoid over-fitting the classification model (Salford Systems, Inc).

[074] The 2D gel electrophoresis data were initially analyzed using Student's t-test, and was performed on two separate classes of data derived from the 635 protein spots that were quantified. The first was performed on the ratio of SNO signal (Asc-) and abundance signal (Asc+); this ratio normalized the SNO signal (Asc-) against the protein abundance indicator (Asc+) for each protein spot. Of the 635 spots detected by 2D gel electrophoresis, 23 had significant p-values ( $< 0.05$ ) for Asc-/Asc+. The second class of data considered only the change in protein abundance (Asc+) between CCM- and CCM+ patients. From this analysis, 13 spots exhibited significant p-values between CCM- and CCM+ patients. Between the two classes of data, the common spots were spot 267, spot 650, and spot 735. The 33 spots in total were used to create a classification model using MARS.

[075] The inventors used the log2 transform of all the data present in the modeling process, with a possible maximum of 15 basis functions and 1 possible interaction term. This resulted in a model with 6 variables whose relative importance to the model is shown in Fig 2, where spot 563 (100%) was the most influential and spot 385 the least. This model resulted in 100% accuracy for classifying CCM+/CCM- patients. The distributions of each spot selected in the MARS model are shown in the box plots (FIGs. 5-10).

[076] The MARS model was unable to produce a highly accurate classifier of CCM+. The resultant model was able to accurately predict CCM- status with 100% accuracy, but only 78% accuracy for the CCM+ patients. This resulted in an overall accuracy of 89%. The AUC (area under the curve) for such a model is 0.99 (FIG. 3).

[077] The optimal MARS model is represented by 6 basis functions. The resultant classification model for these 6 basis functions is  $Y = 1.03427 + 0.586711 \cdot \text{BF2} - 0.178427 \cdot \text{BF5} - 0.638033 \cdot \text{BF10} - 0.263085 \cdot \text{BF11} - 0.244576 \cdot \text{BF12} + 2.64392 \cdot \text{BF14}$  (Table I).

[078] The optimal Stochastic Search Variable Selection (SSVS) model with spike and slab priors identified spots 506 and 758 to differentiate CCM+ from CCM- patients. The

marginal probability of each spot of being selected is shown in the probability plot (FIG. 11). The distributions of protein expression by disease classification are shown in the box plots (FIGs. 12 and 13). The Gibbs sampler method for SSVS allows for the interrogation of the model space efficiently without fitting all possible models with the inferences not driven by the model assumptions. The Bayesian hierarchical methods to variable selection and classification are complementary approaches of omic data in that the uncertainty in the model choice can be incorporated into the analysis. For analysis of the data with 33 spots, the 25,000 iterations of the MCMC were completed by removing the first 1000 for burn-in and saving every 5<sup>th</sup> iteration. In the case of a binary outcome, the latent probit model in which posteriors distribution for the latent indicator variables are estimated via MCMC could be used.

[079] The positions of the MARS and the SSVS selected spots are shown in the SameSpots selected reference gel (FIG. 4), with each spot showing its identity and its selection criterion (either abundance or SNO). The protein IDs and other quantitative information are shown in Table II.

[080] Evaluation of the model performance is seen by analysis of the area under the Receiver Operating Characteristic (ROC) curve (AUC), where sensitivity vs. 1-specificity was plotted. In the ROC analysis, a diagonal line (45 degree slope) starting at zero indicates that the output was a random guess, whereas an ideal classifier with a high true positive rate and low false positive rate will curve positively and strongly towards the upper left quadrant of the plot ( Fawcett, (2006) *Pattern Recognition Letters* 27, 861-74). The AUC is equivalent to the probability that two cases, one chosen at random from each group, are correctly ordered by the classifier ( Hanley and McNeil, (1982) *Radiology* 143, 29-36). The AUC for the CCM+ model is 0.998.

Table I MARS Basis Functions. Shown are the basis functions (BF) for the MARS model for chagasic cardiomyopathy where,  $B_m$  = each individual basis function,  $a_m$  = coefficient of the basis function,  $(y)_+ = \max(0, y)$ . The actual model is:  $Y = 1.03427 + 0.586711*BF2 - 0.178427*BF5 - 0.638033*BF10 - 0.263085*BF11 - 0.244567*BF12 + 2.64392*BF14$ , where  $BF2 = \max(0, 1.07265 - \text{Spot } 385)$ ,  $BF5 = \max(0, \text{Spot } 572 - 0.721195)$ ,  $BF10 = \max(0, 1.9067 - \text{Spot } 563)$ ,  $BF11 = \max(0, \text{Spot } 627 - 0.288306)$ ,  $BF12 = \max(0, \text{Spot } 524 - 0.248617)$ , and  $BF14 = \max(0, 0.74921 - \text{Spot } 411)$

| $B_m$       | Definition                    | $a_m$   | Variable descriptor                 |
|-------------|-------------------------------|---------|-------------------------------------|
| <b>BF2</b>  | $(1.07 - \text{Spot } 385)_+$ | 5.87E-1 | Spot 385 (vimentin-abundance)       |
| <b>BF5</b>  | $(\text{Spot } 572 - 0.72)_+$ | 1.78E-1 | Spot 572 (ferritin light chain-SNO) |
| <b>BF10</b> | $(1.09 - \text{Spot } 563)_+$ | 6.38E-1 | Spot 563 (gamma actin-abundance)    |
| <b>BF11</b> | $(\text{Spot } 627 - 0.29)_+$ | 2.63E-1 | Spot 627 (annexin A6-SNO)           |
| <b>BF12</b> | $(\text{Spot } 524 - 0.25)_+$ | 2.45E-1 | Spot 524 (keratin 10-abundance)     |
| <b>BF14</b> | $(0.75 - \text{Spot } 411)_+$ | 2.64    | Spot 411 (myosin fragment-SNO)      |

Table II Spot identities obtained by mass spectrometry. Both abundance and normalized SNO classifiers are provided. t-test values refer to either abundance or SNO ratios, whichever is provided. SSVS denotes spots were selected by Stochastic Search Variables Selection, not t-test. Identities with MS Scores greater than 56 have significance levels of identification greater than 95%.

| No. | Protein name                 | SwissProt Accession | pI   | MW (kDa) | MS Score | Abundance CCM+:CCM- | t test (p) | CCM+ SNO Ratio | CCM- SNO Ratio |
|-----|------------------------------|---------------------|------|----------|----------|---------------------|------------|----------------|----------------|
| 1   | Vimentin                     | F5H288              | 5.72 | 29       | 171      | 1.43                | 0.006      |                |                |
| 2   | Ferritin-light chain         | PO2792              | 5.80 | 18       | 74       |                     | 0.023      | -1.04          | 1.52           |
| 3   | $\gamma$ -Actin              | I3L1U9              | 5.99 | 18       | 137      | -1.82               | 0.031      |                |                |
| 4   | Annexin A6                   | E5RIU8              | 5.47 | 15       | 103      |                     | 0.040      | -1.18          | 1.30           |
| 5   | Keratin 10                   | P13645              | 9.34 | 19       | 286      | 1.54                | 0.046      |                |                |
| 6   | Myosin-IXa fragment          |                     | 7.98 | 26       | 46       |                     | 0.038      | -1.11          | 1.15           |
| 7   | Parathyroid hormone receptor | H7C0B0              | 8.92 | 20       | 42       |                     | SSVS       | 1.36           | -1.15          |
| 8   | $\beta$ -Actin               | B4E335              | 5.44 | 38       | 624      |                     | SSVS       | -1.02          | -1.23          |

Table III Summary of proteins selected.

## SUMMARY OF PROTEINS SELECTED BY P-VALUE AND RATIOS (ABUNDANCE OR RoR)

| No. | Protein name                                            | Accession No. | pI (Gel) | MW (kD) (Gel) | MS ID Expectation Value | Abundance Ratio | p-Value (Asc+ <0.05) | Ratio of Ratios | p-Value (Asc- <0.05) | Selection Criterion |
|-----|---------------------------------------------------------|---------------|----------|---------------|-------------------------|-----------------|----------------------|-----------------|----------------------|---------------------|
| 1   | Vinculin                                                | P18208        | 7.53     | 89            | 1.9805E-19              | 1.32            | 0.182504             | -1.32           | 0.044584             | RoR                 |
| 2   | Serum albumin (Fragment)                                | H0VA55        | 5.97     | 63            | 1.5811E-12              | -1.25           | 0.204533             | 1.09            | 0.033111             |                     |
| 3   | Isoform 3 of Integrin alpha-1b                          | P08514-3      | 4.19     | 55            | 1.2558E-22              | 1.17            | 0.338162             | -1.08           | 0.027258             |                     |
| 4   | Isoform H7 of Myeloperoxidase                           | P05104-3      | 9.41     | 55            | 9.9763E-04              | 1.75            | 0.125809             | -1.71           | 0.045972             |                     |
| 5   | Actin, cytoplasmic 2, N-terminally processed            | F5H0N0        | 6.41     | 41            | 1.9905E-08              | 1.30            | 0.034774             | -1.34           | 0.047502             |                     |
| 6   | Talin 1                                                 | Q5TCU8        | 5.18     | 32            | 1.5811E-14              | -1.07           | 0.350146             | 1.28            | 0.034137             |                     |
| 7   | Actin, cytoplasmic 1, N-terminally processed            | B4E335        | 4.68     | 30            | 1.2558E-42              | -1.10           | 0.291819             | 1.17            | 0.006024             |                     |
| 8   | Actin, cytoplasmic 1, N-terminally processed            | B4DW52        | 7.51     | 26            | 3.9716E-09              | 1.13            | 0.116141             | -1.27           | 0.023728             |                     |
| 9   | Unconventional myosin-IXa                               | H3BMM1        | 7.98     | 26            | 1.9905E+00              | -1.02           | 0.516115             | -1.13           | 0.037733             |                     |
| 10  | Peptidyl-prolyl cis-trans isomerase A                   | P68237        | 8.63     | 25            | 7.9245E-07              | -1.10           | 0.188105             | 1.14            | 0.021102             |                     |
| 11  | WD repeat-containing protein 48                         | F8WBC8        | 8.84     | 23            | 1.9905E+00              | 1.37            | 0.413441             | 1.09            | 0.042012             |                     |
| 12  | Keratin, type II cytoskeletal 1                         | P04284        | 7.12     | 20            | 3.8716E-11              | -1.08           | 0.61882              | -1.27           | 0.012075             |                     |
| 13  | Parathyroid hormone 2 receptor (Fragment)               | H7C0B0        | 8.82     | 20            | 5.0000E+00              | -1.33           | 0.051258             | 1.50            | 0.033418             |                     |
| 14  | Proteasome subunit beta type-2                          | P49721        | 7.98     | 19            | 5.0000E-04              | -1.08           | 0.532056             | -1.23           | 0.02477              |                     |
| 15  | Ferritin light chain                                    | P02782        | 5.8      | 18            | 3.1548E-03              | 1.23            | 0.08741              | -1.15           | 0.023404             |                     |
| 16  | Annexin                                                 | E5RIU8        | 5.47     | 15            | 3.9716E-08              | 1.44            | 0.150851             | -1.38           | 0.040421             |                     |
| 17  | Actin, cytoplasmic 1, N-terminally processed            | G5E9R0        | 5.12     | 15            | 3.9716E-15              | 1.06            | 0.915841             | -1.19           | 0.020184             |                     |
| 18  | Keratin, type I cytoskeletal 10                         | P13645        | 7.71     | 15            | 1.5811E+00              | -1.00           | 0.214532             | -1.20           | 0.045743             |                     |
| 19  | Heterogeneous nuclear ribonucleoprotein A1 (Fragment)   | F8W846        | 7.26     | 15            | 5.0000E-08              | -1.47           | 0.016761             | 1.52            | 0.043125             |                     |
| 20  | SH3 domain-binding glutamic acid-rich-like protein 3    | Q8H298        | 4.32     | 10            | 9.9763E-31              | 1.29            | 0.029492             | -1.30           | 0.032468             |                     |
| 21  | Ras-related protein Rap-1b                              | B4DQI8        | 4.4      | 0             | 1.2558E-01              | 1.23            | 0.180346             | -1.22           | 0.040893             |                     |
| 22  | Actin, cytoplasmic 1, N-terminally processed            | B4E335        | 5.44     | 38            | 3.1548E-58              | -1.01           | 0.956162             | 1.15            | 0.037853             |                     |
| 23  | Actin, cytoplasmic 1, N-terminally processed            | B4E335        | 7.47     | 78            | 3.1548E-41              | 1.50            | 0.158319             | -1.44           | 0.02411              |                     |
| 24  | POTE ankyrin domain family member F                     | A5A3E0        | 8.57     | 41            | 6.2648E-08              | 1.39            | 0.01115              | -1.35           |                      |                     |
| 25  | Vimentin                                                | F5H288        | 5.72     | 29            | 6.2648E-13              | 1.43            | 0.00823              | -1.41           |                      |                     |
| 26  | Protein S100-A11                                        | P31949        | 6.9      | 10            | 1.9905E-20              | -1.60           | 0.037919             | 1.19            |                      |                     |
| 27  | Isoform 2 of Fibrinogen alpha chain                     | P02671-2      | 8.84     | 41            | 3.1548E-01              | -1.48           | 0.035493             | 1.04            |                      |                     |
| 28  | Tubulin beta chain                                      | Q5JP53        | 8.33     | 34            | 6.2648E-08              | -1.20           | 0.034097             | 1.19            |                      |                     |
| 29  | Myosin regulatory light chain 12B                       | O14850        | 4.38     | 17            | 7.9245E-28              | -1.19           | 0.047581             | 1.07            |                      |                     |
| 30  | Annexin A3                                              | P12429        | 8.09     | 28            | 9.9763E-51              | -1.41           | 0.027378             | 1.34            |                      |                     |
| 31  | Keratin, type I cytoskeletal 10                         | P13645        | 8.34     | 19            | 1.9905E-24              | 1.55            | 0.046182             | -1.35           |                      |                     |
| 32  | Actin, cytoplasmic 2, N-terminally processed (Fragment) | I3L1U9        | 5.99     | 18            | 1.5811E-09              | -1.83           | 0.030827             | 2.47            |                      |                     |
| 33  | ATP synthase subunit alpha                              | A8K082        | 5.64     | 16            | 2.5058E-05              | 1.27            | 0.038451             | 1.17            |                      | Abundance           |

[081] Certain embodiments include biomarkers identified in a previous study. Parasite persistence and oxidative damage in the heart are known to be of pathological significance during Chagas disease (Zacks et al., (2005) *An Acad Bras Cienc*, 77, 695-715; Gupta et al., (2009) *Interdiscip Perspect Infect Dis*, 2009,190354). Infected rats treated with an anti-parasite drug (BZ) and/or antioxidant (PBN) showed that the beneficial effects of these treatments in controlling parasite- and oxidative stress-induced pathology, respectively (Wen et al., (2006) *Am J Pathol*, 169,1953-1964; Wen et al., (2010) *J Am Coll Cardiol*, 55, 2499-2508), are reflected in a plasma proteome profile of chagasic rats. In the previous study the inventors identified 92 proteins that were differentially expressed or oxidized in chagasic plasma. Functional analysis allocated a majority of these proteins to inflammation/immunity and lipid metabolism categories, and to molecular pathways associated with cardiovascular dysfunction, e.g., myocardial infarction, hypertrophy, and fibrosis, and pulmonary embolism and hypertension. Some proteins in chagasic rats treated with PBN and/or BZ were allocated to curative pathways (immune regulation and cardiac remodeling). The 2D-GE results were validated by Western blotting. It was demonstrated that the disease-associated increased expression of GSN and VIM, and release of cardiac MYL2 in the plasma of chagasic rats was normalized by PBN/BZ treatment. Increased plasma levels of GSN, MYL2, and VIM were directly correlated with the severity of cardiac disease in human chagasic patients. This is the first study demonstrating that the plasma oxidative and inflammatory response profile and plasma detection of cardiac proteins parallel the pathologic events contributing to Chagas disease development. These findings have utility in diagnosing disease severity and designing suitable therapy for management of human chagasic patients.

[082] *Inflammation/immune response.* Ingenuity Pathway Analysis (IPA) is a highly curated and comprehensive software used for the integration of proteins into networks and pathways with biological meaning (Thomas and Bonchev, (2010). *Hum Genomics*, 4, 353-360). Network analysis of the plasma proteome profile of chagasic rats identified four major sub-networks linked to host response to *T. cruzi* infection and disease development (Table V). The maximal numbers of the differentially expressed plasma proteins (19 proteins) in chagasic rats were associated with antigen presentation and inflammatory response category (Table V) and indicators of persistent inflammation, known to be of pathological significance in Chagas disease (Dhiman et al., (2009) *Clinical and Vaccine Immunology*, 16, 660-666;

Tanowitz et al., (2009) *Prog Cardiovasc Dis*, 51, 524-539; Junqueira et al., (2010) *Expert Rev Mol Med*, 12, e29). Following functional analysis of the inflammation-associated proteins, 12 proteins (APOA1, APOE, C3, CFB, CFH, FGB, GSN, KRT10, PLG, SCG2, SERPINA1, and SERPINC1) were identified as being involved in immune cell trafficking and cell movement of leukocytes, granulocytes, phagocytes, neutrophils, and dendritic and antigen presenting cells. Some of the differentially expressed proteins in inflammation category were associated with activation (APOE, C3, CFH, GC and PLG), chemotaxis (C3, SCG2, SERPINA1, SERPINC1), and infiltration of leukocytes (APOA1, APOE, C3, PLG, KRT10) and neutrophils (C3, CFB, CFH, PLG). Up regulation of APOE, APOA1, APOH, GC and PLG in chagasic plasma was indicative of activation, binding and accumulation of macrophages in the disease state. Of the 19 differentially expressed inflammation-associated proteins, 11 proteins (APOE, C3, CFB, CFH, GSN, KRT10, SCG2, SERPINA1, SERPINC1, GC, and PLG) were carbonylated in chagasic plasma. PBN treatment prevented the oxidative modification of five of these proteins (i.e., CFB, CFH, SERPRINA1, SERPINC1, and PLG). Interestingly, PBN treatment also normalized or regulated the expression of several inflammation-associated proteins, including APOH, APOE, CFH, PLG and SCG2 in acutely infected rats and APOH, GC, GSN and PLG in chronically infected rats; while the expression level of CFB, GSN, C3, and SERPINC1 was partly regulated by PBN in infected rats (Tables IVa-IVb). Other proteins (APOE, C3, KRT3, and SCG2) were exposed to oxidation due to *T. cruzi*-induced, acute oxidative stress, but were normalized in expression and oxidation during the chronic phase.

[083] Treatment of rats with anti-parasite drug (BZ) was not effective in preventing protein carbonylation. These observations indicate that oxidative stress plays an important role in modulating the host immune response against *T. cruzi*. ROS elicit inflammatory cytokines (e.g. TNF- $\alpha$ , IFN- $\gamma$ , IL-1 $\alpha$ ) in cardiomyocytes infected by *T. cruzi* (Gupta et al., (2009) *Free Radio Biol Med*, 47,1414-21; Ba et al., (2010) *J Biol Chem*, 285, 11596-606). Inflammatory pathology was controlled in chronically infected experimental animals and human patients by enhancing the antioxidant status, which was also beneficial in preserving the cardiac function during Chagas disease (Wen et al., (2006) *Am J Pathol*, 169, 1953-1964; Wen et al., (2010) *J Am Coll Cardiol*, 55, 2499-2508; Ba et al., (2010) *J Biol Chem*, 285, 11596-606; Souza et al., (2010) *Mem Inst Oswaldo Cruz*, 105, 746-751). Recent

observations indicate that the mitochondrial release of ROS due to electron transport chain dysfunction and enhanced release of electrons to molecular oxygen is the primary source of oxidative stress in the heart (Wen and Garg, (2008) *J Bioenerg Biomembr*, 40, 587-598).

[084] *Lipid Metabolism.* Seventeen of the differentially expressed proteins in chagasic plasma, i.e., AFM, C4BPA, CACNA1D, DLGAP2, GC, KNG1, MUG1, MYLPF, MY05A, MY05B, PRPH, PZP, RAI14, and SCG2, were allotted to the lipid metabolism/molecular transport/small molecule biochemistry category (Table V) and functionally linked by IPA network analysis to lipid, fatty acid, and carbohydrate metabolism. A majority of the proteins in this category, i.e., ALB, APOA1, APOA4, APOE, APOH, C3, GC, GNAQ, MY05A, PLG, SCD2, SERPINA1, SERPINC1 and VIM, were linked to the synthesis, metabolism, transport, and modification of lipids and fatty acids, and to uptake and release or efflux of lipids, eicosanoids, and cholesterol. PBN/BZ-treated/infected rats exhibited normalization in the expression of 13 of the proteins linked to lipid/fatty acid metabolism (Tables IVa-IVb). These data provide the first indication that lipid/fatty acid metabolism is dysregulated and of pathologic significance in Chagas disease. The observation of increased expression of CEP350 in chagasic rat plasma provides clues to the pathologic mechanism involved in altered lipid/fatty acid metabolism during Chagas disease. CEP350 is a large centrosome-associated protein with a CAP-Gly domain typically found in cytoskeleton-associated proteins (Yan et al., (2006) *Mol Biol Cell*, 17, 634-44; Patel et al., (2005) *J Cell Sci*, 118, 175-86). CEP350 interacts with other centrosomal proteins (e.g. FGFR1) and has been implicated in the mechanisms underlying microtubule anchoring and organization at the centrosome (Yan et al., (2006) *Mol Biol Cell*, 17, 634-44). Interestingly, CEP350 is also shown to alter the activity and sub-cellular compartmentalization of members of the peroxisome proliferator-activated receptors family (PPAR $\alpha$ , PPAR $\beta/\delta$ , and PPAR $\gamma$ ) (Patel et al., (2005) *J Cell Sci*, 118, 175-86) that heterodimerize with retinoid X receptors (RXRs) to function as transcription factors, and play essential roles in the regulation of cell differentiation and lipid/fatty acid metabolism (Qi et al., (2000) *Cell Biochem Biophys*, 32 Spring, 187-204; Szatmari et al., (2007) *Blood*, 110, 3271-80). Besides CEP350, SERPINS and GPT that were up regulated in chagasic plasma and function in inflammatory response/tissue remodeling and amino acid metabolism, respectively, also belong to the

network of proteins regulated by PPARs (Carter and Church, (2009) *PPAR Res*, 2009, 345320; Rakhshandehroo et al., (2010) *PPAR Res*, 2010).

[085] *Cardiovascular disease-associated proteins.* Twenty-four of the differentially expressed proteins, i.e., AFM, ALB, APOA1, APOA4, APOE, APOH, C3, CFB, CFH, FGB, GC, GNAQ, GSN, HPX, ITIH4, KNG1, MYO5A, PLG, SCD2, SCG2, SERPINA1, SERPINC1, SERPINF1, and VIM, were linked to cardiovascular function, skeletal and muscular disorders, and cardiovascular diseases (Table V). Of these, nine proteins (APOA1, APOE, APOH, C3, KNG1, PLG, SCG2, SERPINC1, and SERPINF1) play a functional role in the proliferation of endothelial cells, while several others correlate with angiogenesis (APOE, APOH, PLG, SCG2, SERPINC1, and SERPINF1), thrombosis or thromboembolism (APOE, APOH, CFB, GNAQ, PLG, and SERPINC1), myocardial ischemia and infarction (ALB, C3, FGB, GSN, PLG, APOA1, APOE, PLG, and SERPINC1), and atherosclerosis (APOA1, APOA4, APOE, and PLG) (Diez et al., (2010) *Mol Biosyst*, 6, 289-304). The latter findings indicate that endothelial cell dysfunction plays a role in the progression of Chagas disease and demonstrate that the plasma proteome profile is a useful indicator of clinical disease status.

[086] *Biomarkers of Chagas disease.* An objective of the studies described below was to identify the diagnostic biomarkers of Chagas disease. Western blot analysis using antibodies specific to GSN, MYL2, and VIM validated the 2D-GE plasma profile of chagasic rats and demonstrated that GSN, MYL2, and VIM are indeed increased in the plasma of chagasic rats. There was a direct correlation in plasma levels of GSN, MYL2, and VIM and disease severity in human chagasic patients. Further, PBN/BZ-mediated control of cardiac pathology and preservation of heart contractile function in chagasic rats (Wen et al., (2006) *Am J Pathol*, 169, 1953-64; Wen et al., (2010) *J Am Coll Cardiol*, 55, 2499-508) was associated with normalized plasma levels of GSN, MYL2, and VIM similar to that noted in normal controls. These findings indicate GSN, MYL2, and VIM can be used as protein biomarkers of Chagas disease.

[087] *Pathologic significance of GSN, MYL2, and VIM in cardiovascular diseases.* Besides their importance as diagnostic markers in Chagas disease observed herein, GSN, MYL2, and VIM play a significant role in heart disease. GSN is an actin-binding protein,

and a member of the gelsolin/villin superfamily (Silacci et al., (2004) *Cell Mol Life Sci*, 61, 2614-23), located intracellularly (cytosol, mitochondria) and extracellularly (blood, plasma) (Koya et al., (2000) *J Biol Chem*, 275, 15343-49). It is a key regulator of actin filament assembly and disassembly and is involved in maintaining cell structure and motility (Silacci et al., (2004) *Cell Mol Life Sci*, 61, 2614-23). Increased GSN expression is associated with interstitial fibrosis and inflammation (Oikonomou et al., (2009) *Thorax*, 64, 467-75), likely due to the GSN-mediated destabilization of cytoskeleton and increased movement of platelets and immune infiltrate, and GSN<sup>-/-</sup> mice are shown to develop decreased pulmonary fibrosis and inflammation (Oikonomou et al., (2009) *Thorax*, 64, 467-75). In the heart, GSN catalyzes the disassembly and degradation of myocardial proteins (Yang et al., (2000) *Circulation*, 102, 3046-52), and its increased expression is detected in failing human hearts (Yang et al., (2000) *Circulation*, 102, 3046-52). It has been suggested that GSN interacts with hypoxia inducible factor 1 (HIF1A), a master transcriptional regulator of the cellular and systemic responses to hypoxia, that is known to play an essential role in the pathophysiology of ischemic cardiovascular disease (Richard et al., (2003) *Circulation*, 107, 2227-32).

[088] MYL2 (myosin regulatory light chain 2) is a cardiac-specific protein. MYL2 dimerizes with cardiac myosin beta (or slow) heavy chain, and its phosphorylation by Ca<sup>+</sup> triggers cardiac contractions. Mutations in MYL2 or abnormalities in MYL2 expression are associated with cardiomyopathy (Richard et al., (2003) *Circulation*, 107, 2227-32), heart failure (Poetter et al., (1996) *Nat Genet*, 13, 63-69), and left ventricular hypertrophy and familial hypertrophy (Flavigny et al., (1998) *J Mol Med*, 76, 208-14; Kabaeva et al., (2002) *Eur J Hum Genet*, 10, 741-48). Expression of MYL2 is altered in chagasic hearts (Cunha-Neto et al., (2005) *Am J Pathol*, 167, 305-13) and isolated cardiomyocytes infected by *T. cruzi* (Goldenberg et al., (2009) *Microbes Infect*, 11, 1140-49), and it is suggested that *T. cruzi*-induced immunoglobulin G autoantibodies and delayed type hypersensitivity to cardiac myosin contribute to disease pathogenesis (Leon and Engman, (2001) *Int J Parasitol*, 31, 555-61; Leon et al., (2004) *Infect Immun*, 72, 3410-17). Certain aspects of the described studies provide the first evidence that the plasma release of MYL2 is linked to disease severity in chagasic patients and indicative of the extent of cardiac muscle injury during Chagas disease development.

[089] VIM is a member of the intermediate filament network, and it is primarily expressed by mesenchymal cells and found in connective tissue. Along with microtubules and actin microfilaments, VIM plays an important role in maintaining cell shape, integrity of the cytoplasm, and stabilizing cytoskeletal interactions (Katsumoto et al., (1990) *Biol Cell*, 68, 139-46). Vimentin is also shown to be localized in the carotid artery and heart valves and serves as a target antigen of peripheral and heart-infiltrating T cells during valvular disease (Fae et al., (2008) *J Autoimmun*, 31, 136-41). Increased detection of vimentin in the heart is indicative of a fibrotic process, as infiltrating fibroblasts replace damaged cardiomyocytes in disease conditions and has been identified by proteomic inventory of myocardial proteins in patients with Chagas disease (Teixeira et al., (2006) *Braz J Med Biol Res*, 39, 1549-62). Results obtained through IPA analysis indicated that VIM modulates NOS2 and is indirectly linked to IL-1 $\beta$  and TNF- $\alpha$  expression in the disease state.

[090] It is demonstrated herein that depletion of high-abundance plasma proteins enhanced the protein discovery of low-abundance proteins by 2D-GE. Pathological events, i.e., persistent inflammation and oxidative stress, associated with Chagas heart disease, and the beneficial effects of antioxidant and anti-parasite therapies in preserving the cardiac function, were reflected in the plasma protein profile of experimentally infected rodents. These proteomic studies provide the first indication that lipid/fatty acid metabolism is dysregulated and of pathologic significance in Chagas disease. Importantly, protein biomarkers (GSN, MYL2, VIM, MYH11, VCL, and PLG) were identified that have utility in diagnosing the presence or severity of Chagas disease, and/or identifying the patients at risk of developing clinical symptoms of Chagas disease.

[091] Certain embodiments are directed to methods of detecting Chagas disease in a biological sample, comprising the step of measuring the presence of at least one protein selected from the group consisting of GSN, MYL2, VIM, MYH11, VCL, and PLG in said sample, wherein elevated levels of GSN, MYL2, VIM, MYH11, VCL, and PLG is indicative of Chagas disease in the subject from which the sample was obtained. Generally, the biological sample is a diagnostic sample from a human or non-human animal. Representative samples include but are not limited to a tissue sample, a plasma sample, or a blood sample. Generally, GSN, MYL2, VIM, MYH11, VCL, and PLG may be detected by any assay known to one of ordinary skill in this art. Representative assays include but are not limited to

a Western blot assay, an ELISA assay, an immunofluorescence assay, an immunoprecipitation assay, and a radioimmunoassay. Preferably, the assay determines the concentration of GSN, MYL2, VIM, MYH11, VCL, or PLG in said sample to be  $\geq 50\%$  greater than normal controls. Normal controls are mammals not having cardiac disease or are not at risk of developing heart failure due to chagasic or other etiologies.

[092] In yet another embodiment of the present invention, there is provided a serodiagnostic kit for determining the presence and/or severity of Chagas disease, said kit comprising: (a) the antibody directed against GSN, MYL2, VIM, MYH11, VCL, and/or PLG, wherein the antibody is linked to a reporter molecule; (b) a buffer; and, (c) a reagent for detection of the reporter molecule. Useful antibodies directed against GSN, MYL2, VIM, MYH11, VCL, and PLG are well known to those with ordinary skill in this art. Representative reporter molecules include but are not limited to luciferase, horseradish peroxidase, P-galactosidase, and fluorescent labels.

Table IVa. List of Genes/Proteins Differentially Expressed in Response to *T. Cruzi* Infection

| Spot#                              | Gene Name | Protein Name                        | Accession No. | Putative Biological Function | Putative Cellular Location |
|------------------------------------|-----------|-------------------------------------|---------------|------------------------------|----------------------------|
| 12, 180                            | KRT1      | Keratin, type II                    | gi/120474989  | Oxidative stress res         | Membrane                   |
| 34                                 | TTC37     | KIAA0372 gene product               | gi/149058911  | Protein binding              | N/A                        |
| 37, 593                            | SRPRB     | Bal-667                             | gi/33086638   | Iron homeostasis             | Extracellular              |
| 49, 673                            | IGH-1     | Igh-la protein                      | gi/299352     | Antigen binding              | Extracellular              |
| 52, 54, 302                        | TF        | Transferrin                         | gi/1854476    | Transport                    | Extracellular              |
| 53, 225                            | SroTP     | Serotransferrin                     | gi/61556986   | Proteolysis                  | Membrane                   |
| 72                                 | IDH3A     | Isocitrate dehydrogenase 3          | gi/149041700  | Metabolism                   | Mitochondria               |
| 73, 705, 787                       | ALB       | Alpha-1-inhibitor 3                 | gi/83816939   | Inflammatory respo           | Extracellular              |
| 77                                 | MUG1      | Murinoglobulin-1                    | gi/12831225   | Acute-phase respo            | Extracellular              |
| 88                                 | IGHG-     | g-2a immunoglobulin heav            | gi/1220486    | Antigen binding              | N/A                        |
| 94                                 | FGB       | Fibrinogen beta chain               | gi/124106312  | Signal transductio           | Membrane                   |
| 106                                | CDK5RAP2  | CDK5 regulatory associ<br>prtoein 2 | gi/109476582  | tRNA modification            | Cytoplasm                  |
| 115, 123                           | SERPINAL1 | Alpha-1-antiproteinase              | gi/112889     | Acute phase respo            | Extracellular              |
| 149                                | PUS1      | Pseudouridine synthase 1            | gi/149063707  | tRNA processing              | Mitochondria               |
| 153                                | MYL2      | Myosin, light polypeptid<br>2       | gi/149067749  | Muscle contraction           | Cytosol                    |
| 177                                | AIM       | Alpha-1-macroglobulin pre           | gi/21955142   | Protein binding              | Extracellular              |
| 181                                | SERPINA3L | Serine protease inhibitor A         | gi/2507387    | Inhibitory protein           | Extracellular              |
| 184, 257, 328                      | CFH       | Complement inhibitory fac           | gi/15485713   | Immune response              | Cytoplasm                  |
| 207, 555                           | CCHL1A1   | Calcium channel alpha-1 su          | gi/1184038    | Transport                    | Membrane                   |
| 215                                | GPT       | Glutamic pyruvic<br>transaminase 1  | gi/149066073  | Gluconeogenesis              | Cytoplasm                  |
| 266                                | CEP350    | Centrosome-associated pro           | gi/18027304   | Cytoskeleton                 | Cytoplasm                  |
| 299                                | UMPS      | Uridine monophosphate               | gi/149060638  | Metabolism                   | Cytoplasm                  |
| 315                                | ITIH4     | Inter-alpha-inhibitor H4            | gi/126722991  | Metabolism                   | Extracellular              |
| 334                                | ZNF689    | Zinc finger, HIT type 6             | gi/157818873  | Transcription                | Nucleus                    |
| 341                                | Nmag_2782 | Na7Ca <sup>+</sup> antiporter       | gi/8825638    | Transport                    | Membrane                   |
| 355, 359                           | PLG       | Plasminogen                         | gi/16758216   | Tissue remodeling            | Extracellular              |
| 382, 385,<br>378, 572,<br>578, 595 | CFB       | Complement factor B                 | gill49027999  | Immune response              | Extracellular              |
| 389                                | GSN       | Gelsolin                            | gi/149038928  | Cytoskeleton                 | Cytosol                    |
| 430                                | SCD2      | Stearyl-CoA desaturase 2            | gi/1763027    | Fatty acid synthes           | Membrane                   |
| 434, 678                           | COG1      | Component of golgi cor              | gi/149054700  | Transport                    | Membrane                   |
| 439                                | C4BPA     | C4b-binding protein alp             | gi/2493792    | Innate immunity              | Extracellular              |
| 452                                | AFM       | Afamin, Albumin-bindin              | gi/60688254   | Transport                    | Extracellular              |
| 464                                | IGH-6     | Immunoglobulin heavy                | gi/62201965   | Antigen binding              | N/A                        |
| 479                                | IGHM      | Ig mu chain C region                | gi/111977     | Immune response              | Membrane                   |
| 493                                | ZFP637    | Zinc finger protein 637             | gi/201027426  | Protein binding              | Intracellular              |
| 515                                | CESIC/ES2 | Carboxylesterase                    | gi/468766     | Metabolism                   | Endoplasmic<br>reticulum   |
| 524                                | ERCC4     | DNA repair endonucleas              | gi/109487684  | DNA repair                   | Nucleus                    |
| 551, 587,<br>908                   | C3        | Complement C3                       | gi/158138561  | Inflammation                 | Extracellular              |
| 559                                | HPX       | Hemopexin                           | gi/122065203  | Heme scavenging              | Extracellular              |
| 571                                | GC        | Group specific compone              | gi/51260133   | Vitamin D binding            | Extracellular              |
| 576, 620                           | Ac2-248   | SERPIN-like protein                 | gi/32527753   | Inflammation resp            | Extracellular              |

| Spot#    | Gene Name      | Protein Name                            | Accession No. | Putative Biological Function | Putative Cellular Location |
|----------|----------------|-----------------------------------------|---------------|------------------------------|----------------------------|
| 586, 614 | SERPINC1       | Serine/cysteine peptidase               | gi/58865630   | Blood clotting               | Extracellular              |
| 592      | DNASE1L1       | Deoxyribonuclease 1-like                | gi/149029874  | DNA catabolism               | Endoplasmic reticulum      |
| 604      | APOH           | Beta-2-glycoprotein 1                   | gi/57528174   | Transport                    | Cell surface               |
| 606      | GC             | Vitamin D binding protein               | gi/203927     | Transport                    | Extracellular              |
| 627      | MAPI           | LMW T-kininogen 1                       | gi/205085     | Acute-phase response         | Extracellular              |
| 638      | LOC501738      | Immune activating receptor              | gi/264681503  | Receptor activity            | N/A                        |
| 649      | SYNE2          | Nesprin-2 like                          | gi/109478368  | Cytoskeleton                 | Nuclear                    |
| 651      | DSTN           | Destrin                                 | gi/75991707   | Actin binding                | Cytoplasm                  |
| 655      | DLGAP2         | Disks large-associated protein          | gi/16758774   | Synapse transmission         | Membrane                   |
| 672      | SERPINF        | Serine/cysteine peptidase               | gi/29293811   | Inhibitory protein           | Extracellular              |
| 676      | rCG33447       | Hypothetical                            | gi/149052919  | N/A                          | N/A                        |
| 691      | APOA4          | Apolipoprotein A-IV                     | gi/114008     | Lipid binding                | Extracellular              |
| 860      | KRT10          | Keratin, type I                         | gi/57012436   | Protein binding              | Intermediate filament      |
| 966      | Hypothetical   | CMRF-35-like molecule                   | gi/109511428  | Immunity                     | Membrane                   |
| 897      | MYO5A          | Myosin Va                               | gi/149019170  | Myosin complex               | Cytoplasm                  |
| 715      | SERPINA1       | Proteinase inhibitor-like               | gi/930263     | Protein binding              | Extracellular              |
| 724      | ALB            | Serum albumin                           | gi/158138568  | Transport                    | Extracellular              |
| 728      | SYMPK          | Symplekin-C                             | gi/62531221   | Protein binding              | Nucleus                    |
| 733      | DECR2          | 2,4-dienoyl-CoA reductase               | gi/25282441   | Metabolism                   | Peroxisome                 |
| 740      | PRPH           | Peripherin                              | gi/166063971  | Cytoskeleton                 | Intermediate filament      |
| 745      | PFKFB1         | 6-phosphofructo-2-kinase                | gi/77020248   | Metabolism                   | Cytoplasm                  |
| 749      | MYO5B          | Myosin-Vb                               | gi/8393817    | Myosin complex               | Intracellular              |
| 766      | SCG2           | Secretogranin 2                         | gi/149016236  | Inflammation                 | Extracellular              |
| 779,909  | VIM            | Vimentin                                | gi/149021116  | Cell integrity               | Cytoplasm                  |
| 796      | Hypothetical p |                                         | gi/109468251  | N/A                          | N/A                        |
| 814      | RBM15B         | RNA binding motif 15B                   | gi/109483938  | RNA splicing                 | Nucleoplasm                |
| 817      | BRPF1          | Bromodomain/PHD finger protein 1        | gi/109472470  | Transcription                | Cytoplasm                  |
| 820,1081 | BFAR           | Bifunctional apoptosis re               | gi/61557021   | Apoptosis                    | Membrane                   |
| 827      | GNAT           | Guanine binding protein                 | gi/84662745   | N/A                          | Cytoplasm                  |
| 832      | PSMB           | Proteasome beta-type subunit            | gi/9653292    | Protein catabolism           | Cytoplasm                  |
| 867      | RAI14          | Ankycorbin                              | gi/58865464   | N/A                          | Cytoplasm                  |
| 881      | CHD4           | Chromodomain helicase binding protein 4 | gi/149049419  | Transcription                | Nucleus                    |
| 900      | rCG25416       | Transferrin region                      | gi/149018747  | N/A                          | N/A                        |
| 906      | APOE           | Apolipoprotein E                        | gi/149056721  | Oxidative stress re          | Chylomicron                |
| 979      | rCG25357       |                                         | gi/149018900  | N/A                          | N/A                        |
| 992      | APOA1          | Apolipoprotein A-I                      | gi/2145143    | Transport                    | Membrane                   |
| 1019     | ZNHIT6         | Hypothetical protein FL                 | gi/149026162  | Protein binding              | Pre-snoRNP complex         |

Table IVb. Differential Expression in Response to T. Cruzi Infection

| Spot#                        | MW (Da)  | pI   | e-value<br>p<0.001 | RN*   | RA*   | RAP*  | RC*   | RCP*  | RCB*  | RCPB* |
|------------------------------|----------|------|--------------------|-------|-------|-------|-------|-------|-------|-------|
| 12, 180                      | 65059.2  | 8.04 | 9.10E-09           | 4.70  | 4.03  | 21.47 | 9.92  | 7.22  | 9.47  | 11.17 |
| 34                           | 68849.3  | 6.22 | 0.001              | 2.29  | 0.64  | 0.37  | 0.31  | 0.45  | 0.37  | 0.25  |
| 37, 593                      | 109545.9 | 8.35 | 5.31E-08           | 2.14  | 5.33  | 14.38 | 6.46  | 6.48  | 3.21  | 8.61  |
| 49, 673                      | 52500    | 7.23 | 0.132              | 7.89  | 9.90  | 19.12 | 50.91 | 13.39 | 3.98  | 4.48  |
| 52, 54, 302                  | 78538.3  | 6.94 | 5.55E-08           | 9.83  | 5.80  | 9.80  | 2.85  | 14.45 | 0.60  | 8.06  |
| 53, 225                      | 78512.5  | 7.14 | 1.671E-07          | 6.78  | 6.20  | 8.76  | 2.05  | 15.12 | 1.72  | 5.60  |
| 72                           | 30334.3  | 5.83 | 6.694E-07          | 39.31 | 10.26 | 18.10 | 76.03 | 16.43 | 83.09 | 13.00 |
| 73, 705, 787                 | 165038.2 | 5.7  | 6.561E-09          | 86.41 | 19.56 | 46.94 | 136.2 | 38.67 | 216.9 | 28.67 |
| 77                           | 166589.9 | 5.68 | 0.00007856         | 28.44 | 16.28 | 16.3  | 47.41 | 8.675 | 107.7 | 20.1  |
| 88                           | 52242.6  | 8.15 | 1.24E-08           | 2.54  | 1.45  | 74.61 | 38.51 | 40.49 | 4.75  | 17.20 |
| 94                           | 54827.9  | 7.9  | 0.0008907          | 0.33  | 0.51  | 1.84  | 1.87  | 2.55  | 0.55  | 0.53  |
| 106                          | 208168.7 | 5.27 | 0.001              | 1.47  | 0.85  | 1.70  | 2.22  | 6.94  | 2.21  | 1.67  |
| 115, 123                     | 46277.6  | 5.7  | 0.272              | 11.10 | 6.35  | 22.27 | 10.54 | 10.96 | 8.36  | 18.61 |
| 149                          | 44463.4  | 8.1  | 0.000911           | 6.34  | 1.67  | 0.78  | 6.50  | 4.39  | 1.16  | 1.17  |
| 153                          | 17669.7  | 4.96 | 0.00004491         | 9.21  | 3.03  | 27.07 | 38.86 | 10.34 | 2.37  | 2.66  |
| 177                          | 168421.9 | 6.46 | 2.312E-10          | 2.88  | 1.84  | 3.71  | 28.06 | 1.60  | 2.77  | 2.87  |
| 181                          | 46419.1  | 5.48 | 0.007              | 1.83  | 3.57  | 3.99  | 17.08 | 1.90  | 7.53  | 3.22  |
| 184, 257, 328                | 144813.9 | 6.52 | 0.001              | 3.69  | 9.51  | 4.54  | 2.46  | 5.85  | 6.15  | 3.32  |
| 207, 555                     | 13893.9  | 6.52 | 0.0001206          | 15.79 | 10.78 | 13.23 | 13.97 | 9.12  | 11.32 | 18.65 |
| 215                          | 50475.5  | 6.63 | 2.201E-08          | 3.47  | 1.20  | 1.73  | 26.17 | 1.97  | 3.23  | 1.98  |
| 266                          | 166809.6 | 8.63 | 0.012              | 0.56  | 0.22  | 0.75  | 0.56  | 0.54  | 1.43  | 1.22  |
| 299                          | 33345.2  | 6.02 | 0.00001753         | 2.05  | 1.45  | 1.92  | 0.66  | 1.17  | 1.17  | 2.01  |
| 315                          | 103861.8 | 5.82 | 0.00001561         | 1.93  | 2.95  | 6.24  | 7.00  | 8.23  | 5.19  | 3.92  |
| 334                          | 53374.8  | 5.51 | 4.73E-08           | 26.07 | 0.59  | 0.61  | 1.00  | 0.71  | 1.05  | 0.45  |
| 341                          | 11217.6  | 8.91 | 5.217E-06          | 1.87  | 1.50  | 0.59  | 1.10  | 1.10  | 1.44  | 6.59  |
| 355, 359                     | 93213.9  | 6.79 | 0.00001998         | 3.15  | 4.34  | 2.99  | 3.15  | 2.20  | 3.29  | 3.45  |
| 382, 385, 378, 572, 578, 595 | 83735.6  | 6.05 | 1.675E-10          | 5.91  | 43.78 | 33.76 | 30.02 | 3.29  | 23.45 | 5.94  |
| 389                          | 86314.2  | 5.76 | 6.661E-14          | 6.51  | 70.05 | 37.55 | 38.06 | 4.32  | 44.17 | 8.39  |

| Spot#         | MW (Da)  | pI   | e-value<br>p<0.001 | RN*   | RA*   | RAP*  | RC*    | RCP*   | RCB*   | RCPB*  |
|---------------|----------|------|--------------------|-------|-------|-------|--------|--------|--------|--------|
| 430           | 3479.7   | 9.9  | 2.661 E-09         | 11.28 | 40.97 | 22.40 | 9.29   | 8.21   | 13.65  | 11.38  |
| 434, 678      | 72098.8  | 8.06 | 1.465E-08          | 29.10 | 110.5 | 55.14 | 34.52  | 24.63  | 43.20  | 30.09  |
| 439           | 64277.8  | 7.06 | 9.889E-09          | 4.21  | 1.72  | 4.04  | 13.64  | 14.99  | 1.36   | 7.56   |
| 452           | 54205.3  | 5.8  | 5.923E-09          | 25.61 | 13.68 | 18.92 | 17.57  | 10.01  | 59.65  | 55.78  |
| 464           | 69059.5  | 5.69 | 0.0004813          | 5.89  | 7.22  | 3.89  | 3.22   | 6.65   | 4.66   | 7.97   |
| 479           | 38189.1  | 6.72 | 1.038E-11          | 2.72  | 21.09 | 23.96 | 10.08  | 32.56  | 8.65   | 14.62  |
| 493           | 31712.4  | 9.5  | 6.464E-07          | 3.75  | 14.49 | 9.82  | 5.45   | 13.46  | 17.41  | 12.88  |
| 515           | 59196.9  | 5.51 | 5.223E-07          | 17.08 | 3.47  | 6.87  | 8.05   | 7.78   | 24.22  | 19.57  |
| 524           | 110025.1 | 9.2  | 9.067E-09          | 120.8 | 32.57 | 53.74 | 55.47  | 21.96  | 68.54  | 30.55  |
| 551, 587, 908 | 187745.9 | 6.06 | 1.64E-11           | 7.046 | 146.9 | 88.04 | 46.76  | 54.1   | 32.88  | 31.97  |
| 559           | 52059.6  | 7.58 | 0.167              | 26.14 | 16.11 | 24.39 | 38.23  | 27.58  | 17.91  | 22.98  |
| 571           | 55079.6  | 5.65 | 9.36E-12           | 37.07 | 11.96 | 7.84  | 74.44  | 38.63  | 3.75   | 24.61  |
| 576, 620      | 67191    | 6.85 | 1.098E-07          | 10.86 | 14.95 | 18.08 | 16.62  | 16.66  | 2.38   | 11.23  |
| 586, 614      | 52714    | 6.18 | 3.838E-06          | 63.42 | 48.70 | 50.44 | 57.02  | 38.00  | 132.20 | 75.38  |
| 592           | 32437    | 6.31 | 1.664E-07          | 21.16 | 20.04 | 15.61 | 12.37  | 31.55  | 11.70  | 17.68  |
| 604           | 39743.2  | 8.58 | 6.515E-06          | 18.54 | 10.78 | 22.81 | 17.48  | 23.66  | 67.12  | 26.76  |
| 606           | 55089.6  | 5.65 | 0.006              | 15.31 | 5.92  | 9.60  | 9.50   | 5.58   | 11.27  | 10.14  |
| 627           | 48757    | 6.29 | 1.263E-07          | 108.9 | 20.28 | 30.23 | 45.81  | 54.85  | 44.92  | 38.20  |
| 638           | 24023.2  | 9.41 | 0.00001901         | 3.11  | 4.73  | 4.42  | 5.82   | 5.92   | 4.69   | 12.14  |
| 649           | 442636.4 | 5.29 | 1.843E-08          | 52.04 | 10.43 | 40.69 | 33.92  | 37.11  | 26.48  | 25.27  |
| 651           | 18806.7  | 8.19 | 3.884E-10          | 23.61 | 109.0 | 94.03 | 176.80 | 57.85  | 37.12  | 51.53  |
| 655           | 111348.9 | 6.82 | 6.158E-10          | 48.96 | 280.6 | 240.6 | 254.70 | 149.20 | 151.30 | 122.60 |
| 672           | 46493.2  | 6.04 | 0.00003611         | 9.02  | 3.59  | 7.34  | 3.45   | 17.65  | 23.29  | 10.52  |
| 676           | 20157.4  | 9.14 | 0.00001667         | 13.09 | 19.5  | 14.69 | 49.61  | 42.59  | 24.67  | 17.95  |
| 691           | 44428.7  | 5.12 | 1.996E-06          | 46.70 | 42.11 | 49.99 | 39.11  | 16.29  | 65.41  | 14.52  |
| 860           | 56698.6  | 5.1  | 4.594E-08          | 6.32  | 2.94  | 3.06  | 3.63   | 5.79   | 2.64   | 20.82  |
| 966           | 9215.8   | 7.93 | 3.917E-09          | 12.66 | 76.58 | 53.33 | 87.53  | 59.29  | 49.99  | 28.90  |
| 897           | 96579.8  | 9.48 | 5.875E-06          | 24.00 | 9.21  | 4.82  | 16.65  | 31.94  | 11.65  | 19.24  |
| 715           | 22867.8  | 6.06 | 2.851E-07          | 6.35  | 12.36 | 10.83 | 24.11  | 32.20  | 30.96  | 12.93  |
| 724           | 70709.9  | 6.09 | 5.902E-07          | 1.19  | 3.31  | 1.72  | 0.46   | 2.23   | 2.19   | 11.17  |
| 728           | 33433.1  | 4.97 | 1.601E-11          | 0.84  | 1.00  | 0.76  | 0.49   | 1.13   | 2.21   | 7.89   |
| 733           | 31614.4  | 8.51 | 0.0006324          | 1.71  | 1.90  | 2.24  | 2.06   | 2.44   | 1.67   | 7.42   |

| Spot#     | MW (Da)  | pI   | e-value<br>p<0.001 | RN*   | RA*   | RAP*  | RC*   | RCP*  | RCB*  | RCPB* |
|-----------|----------|------|--------------------|-------|-------|-------|-------|-------|-------|-------|
| 740       | 54063.5  | 5.32 | 0.0001771          | 14.90 | 5.56  | 7.81  | 4.46  | 7.33  | 4.02  | 13.03 |
| 745       | 55301    | 6.78 | 3.474E-08          | 8.56  | 4.94  | 4.51  | 4.17  | 3.21  | 2.30  | 8.93  |
| 749       | 215240.6 | 6.53 | 6.828E-10          | 5.53  | 9.51  | 4.71  | 2.88  | 1.70  | 2.46  | 19.91 |
| 766       | 61578.7  | 4.69 | 0.000699           | 3.94  | 5.60  | 3.20  | 3.16  | 6.32  | 3.79  | 14.46 |
| 779, 909  | 4131.2   | 9.75 | 2.984E-09          | 1.52  | 6.85  | 5.45  | 36.40 | 6.88  | 3.08  | 7.28  |
| 796       | 53156.7  | 8.32 | 1.702E-07          | 10.25 | 8.42  | 9.97  | 7.48  | 8.33  | 5.11  | 11.64 |
| 814       | 96223.1  | 9.85 | 4.256E-07          | 1.65  | 1.41  | 0.60  | 0.63  | 1.56  | 3.61  | 13.27 |
| 817       | 151416.4 | 8.55 | 0.024              | 10.16 | 21.83 | 12.14 | 8.66  | 29.59 | 25.15 | 31.35 |
| 820, 1081 | 53617.2  | 6.44 | 0.006              | 4.77  | 3.19  | 4.59  | 31.17 | 2.20  | 13.38 | 5.61  |
| 827       | 42416.4  | 5.48 | 4.658E-09          | 11.89 | 15.94 | 17.91 | 3.82  | 17.80 | 10.78 | 12.84 |
| 832       | 15426.5  | 6.82 | 0.014              | 2.47  | 3.16  | 6.23  | 1.86  | 7.55  | 3.47  | 8.94  |
| 867       | 106502   | 5.64 | 0.0002925          | 4.34  | 5.72  | 2.56  | 2.86  | 3.00  | 2.41  | 12.07 |
| 881       | 111042   | 5.43 | 2.167E-10          | 11.78 | 5.61  | 13.92 | 17.58 | 6.68  | 28.89 | 8.62  |
| 900       | 66991.6  | 6.41 | 9.426E-08          | 20.46 | 9.26  | 25.94 | 22.29 | 44.00 | 7.06  | 18.89 |
| 906       | 27354.1  | 7.93 | 0.004              | 43.97 | 56.73 | 49.40 | 26.45 | 87.74 | 74.42 | 57.50 |
| 979       | 3285.6   | 9.5  | 2.003E-07          | 20.05 | 55.99 | 59.46 | 45.31 | 41.38 | 31.72 | 31.72 |
| 992       | 29869.1  | 5.51 | 8.784E-07          | 15.55 | 41.44 | 34.51 | 32.12 | 57.94 | 38.11 | 39.05 |

N/A: No match available in public information databases, pI: Isoelectric pH, MW: molecular weight

\* Average Normalized Density x 100,000

Table V IPA network analysis of differentially expressed plasma proteins in Chagasic rats

| ID | Molecules in Network                                                                                                                                                                                                                                                               | Score | Focus Molecules | Top Functions                                                        |
|----|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------|-----------------|----------------------------------------------------------------------|
| 1  | Actin, ALB, APOA1, APOA4, APOE, APOH, C3, CFB, CFH, CFHR1, CFP, CHD4, Cytokeratin, DSTN, FGB, Fibrinogen, GSN, HPX, IgG, KRT1, KRT3, KRT4, KRT10, KRT12, KRT13, KRT23, KRT6B, PLG, SERPINF, SERPINF1, SETX, TF, TMPRSS6, TRY6, VIM                                                 | 41    | 19              | Antigen Presentation, Humoral Immune Response, Inflammatory Response |
| 2  | AFM, APP, BRPF3, C4BPA, CACNA1D, COL25A1, DLGAP2, GAB1/2, GC, GRB2, heparin, INSR, KCNMA1, KNG1 (includes EG:16644), Met dimer, MUG1, MYLPP, MYO5A, MYO5B, MYO5C, PFKFB2, PIK3AP1, PIK3R1, PLA2G2D, PLA2G2E, PRPH, PZP, RAI14, SCG2, SERPINC1, SLC23A1, SNX8, VPS13A, YWHAZ, ZNF32 | 36    | 17              | Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry   |
| 3  | ATG4C, BFA, C10RF25, CASP8, CEBPB, CEP350, CIDEA, CRAT, DSCR3, ESR1, GNAQ, GPT, HNF4A, IDH3A, ITIH4, LGMN, MINA, MYC, PEPD, PPARG, PUS1, PUS3, SCD2, SERPINA1, SLC25A19, SQDRL (includes EG:58472), SRPRB, SYMPK, TMEM176A, TMEM176B, TRUB2, TTC37, UMPS, YME1L1, ZNHIT6           | 29    | 14              | Cellular Development, Free Radical Scavenging, Molecular Transport   |

### I. Biomarkers

[093] Biomarkers can be used to both define a disease state as well as to provide a means to predict physiological and clinical manifestations of a disease. Three commonly discussed ways in which biomarkers can be used clinically are: (1) to characterize a disease state, i.e. establish a diagnosis, (2) to demonstrate the progression of a disease, and (3) to predict the progression of a disease, i.e. establish a prognosis. Establishing putative biomarkers for such uses typically requires a statistical analysis of relative changes in biomarker expression either cross-sectionally and/or over time (longitudinally). For example, in a state or diagnostic biomarker analysis, levels of one or more biomarkers are measured cross-sectionally, e.g. in patients with disease and in normal control subjects, at one point in time and then related to the clinical status of the groups. Statistically significant differences in biomarker expression can be linked to presence or absence of disease, and would indicate that the biomarkers could subsequently be used to diagnose patients as either having disease

or not having disease. In a progression analysis, levels of one or more biomarkers and clinical status are both measured longitudinally. Statistically significant changes over time in both biomarker expression and clinical status would indicate that the biomarkers under study could be used to monitor the progression of the disease. In a prognostic analysis, levels of one or more biomarkers are measured at one point in time and related to the change in clinical status from that point in time to another subsequent point in time. A statistical relationship between biomarker expression and subsequent change in clinical status would indicate that the biomarkers under study could be used to predict disease progression.

[094] Results from prognostic analyses can also be used for disease staging and for monitoring the effects of drugs. The prediction of variable rates of decline for various groups of patients allows them to be identified as subgroups that are differentiated according to disease severity (i.e. less versus more) or stage (i.e. early versus late). Also, patients treated with a putative disease-modifying therapy may demonstrate an observed rate of decline that does not match the rate of decline predicted by the prognostic analysis. This could be considered evidence of drug or treatment efficacy.

## II. Computer Implementation

[095] Embodiments of assays described herein or the analysis thereof may be implemented or executed by one or more computer systems. One such computer system is illustrated in FIG. 12. In various embodiments, computer system may be a server, a mainframe computer system, a workstation, a network computer, a desktop computer, a laptop, or the like. For example, in some cases, the analysis described herein or the like may be implemented as a computer system. Moreover, one or more of servers or devices may include one or more computers or computing devices generally in the form of a computer system. In different embodiments these various computer systems may be configured to communicate with each other in any suitable way, such as, for example, via a network.

[096] As illustrated, the computer system includes one or more processors 510 coupled to a system memory 520 via an input/output (I/O) interface 530. Computer system 500 further includes a network interface 540 coupled to I/O interface 530, and one or more input/output devices 550, such as cursor control device 560, keyboard 570, and display(s) 580. In some embodiments, a given entity (e.g., analysis of subjects for trypanosome

infection and/or cardiomyopathy) may be implemented using a single instance of computer system 500, while in other embodiments multiple such systems, or multiple nodes making up computer system 500, may be configured to host different portions or instances of embodiments. For example, in an embodiment some elements may be implemented via one or more nodes of computer system 500 that are distinct from those nodes implementing other elements (*e.g.*, a first computer system may implement an assessment of a hybrid latent variable assessment or system while another computer system may implement data gathering, scaling, classification etc.).

[097] In various embodiments, computer system 500 may be a single-processor system including one processor 510, or a multi-processor system including two or more processors 510 (*e.g.*, two, four, eight, or another suitable number). Processors 510 may be any processor capable of executing program instructions. For example, in various embodiments, processors 510 may be general-purpose or embedded processors implementing any of a variety of instruction set architectures (ISAs), such as the x86, POWERPC®, ARM®, SPARC®, or MIPS® ISAs, or any other suitable ISA. In multi-processor systems, each of processors 510 may commonly, but not necessarily, implement the same ISA. Also, in some embodiments, at least one processor 510 may be a graphics-processing unit (GPU) or other dedicated graphics-rendering device.

[098] System memory 520 may be configured to store program instructions and/or data accessible by processor 510. In various embodiments, system memory 520 may be implemented using any suitable memory technology, such as static random access memory (SRAM), synchronous dynamic RAM (SDRAM), nonvolatile/Flash-type memory, or any other type of memory. As illustrated, program instructions and data implementing certain operations, such as, for example, those described herein, may be stored within system memory 520 as program instructions 525 and data storage 535, respectively. In other embodiments, program instructions and/or data may be received, sent or stored upon different types of computer-accessible media or on similar media separate from system memory 520 or computer system 500. Generally speaking, a computer-accessible medium may include any tangible storage media or memory media such as magnetic or optical media—*e.g.*, disk or CD/DVD-ROM coupled to computer system 500 via I/O interface 530. Program instructions and data stored on a tangible computer-accessible medium in non-transitory form may further

be transmitted by transmission media or signals such as electrical, electromagnetic, or digital signals, which may be conveyed via a communication medium such as a network and/or a wireless link, such as may be implemented via network interface 540.

[0099] In an embodiment, I/O interface 530 may be configured to coordinate I/O traffic between processor 510, system memory 520, and any peripheral devices in the device, including network interface 540 or other peripheral interfaces, such as input/output devices 550. In some embodiments, I/O interface 530 may perform any necessary protocol, timing or other data transformations to convert data signals from one component (*e.g.*, system memory 520) into a format suitable for use by another component (*e.g.*, processor 510). In some embodiments, I/O interface 530 may include support for devices attached through various types of peripheral buses, such as a variant of the Peripheral Component Interconnect (PCI) bus standard or the Universal Serial Bus (USB) standard, for example. In some embodiments, the function of I/O interface 530 may be split into two or more separate components, such as a north bridge and a south bridge, for example. In addition, in some embodiments some or all of the functionality of I/O interface 530, such as an interface to system memory 520, may be incorporated directly into processor 510.

[0100] Network interface 540 may be configured to allow data to be exchanged between computer system 500 and other devices attached to a network, such as other computer systems, or between nodes of computer system 500. In various embodiments, network interface 540 may support communication via wired or wireless general data networks, such as any suitable type of Ethernet network, for example; via telecommunications/telephony networks such as analog voice networks or digital fiber communications networks; via storage area networks such as Fiber Channel SANs, or via any other suitable type of network and/or protocol.

[0101] Input/output devices 550 may, in some embodiments, include one or more display terminals, keyboards, keypads, touch screens, scanning devices, voice or optical recognition devices, or any other devices suitable for entering or retrieving data by one or more computer system 500. Multiple input/output devices 550 may be present in computer system 500 or may be distributed on various nodes of computer system 500. In some embodiments, similar input/output devices may be separate from computer system 500 and may interact with one or

more nodes of computer system 500 through a wired or wireless connection, such as over network interface 540.

[0102] As shown in FIG. 14, memory 520 may include program instructions 525, configured to implement certain embodiments described herein, and data storage 535, comprising various data accessible by program instructions 525. In an embodiment, program instructions 525 may include software elements of embodiments illustrated herein. For example, program instructions 525 may be implemented in various embodiments using any desired programming language, scripting language, or combination of programming languages and/or scripting languages (*e.g.*, C, C++, C#, JAVA®, JAVASCRIPT®, PERL®, etc). Data storage 535 may include data that may be used in these embodiments. In other embodiments, other or different software elements and data may be included.

[0103] A person of ordinary skill in the art will appreciate that computer system 500 is merely illustrative and is not intended to limit the scope of the disclosure described herein. In particular, the computer system and devices may include any combination of hardware or software that can perform the indicated operations. In addition, the operations performed by the illustrated components may, in some embodiments, be performed by fewer components or distributed across additional components. Similarly, in other embodiments, the operations of some of the illustrated components may not be performed and/or other additional operations may be available. Accordingly, systems and methods described herein may be implemented or executed with other computer system configurations.

## CLAIMS

1. A method of assessing a subject having Chagas disease comprising: measuring levels of (i) one or more proteins selected from vimentin, gamma actin, or keratin 10, or (ii) level of cysteinyl-S-nitrosylation (SNO) of one or more of beta actin, annexin A6, ferritin light chain fragment (~18kDa), parathyroid hormone 2 receptor fragment (~20kDa), or myosin-IXa fragment; wherein elevated levels of the proteins or nitrosylation is indicative of risk for developing chagasic cardiomyopathy.
2. The method of claim 1, wherein the levels of vimentin, gamma actin, and keratin 10 are measured.
3. The method of claim 2, wherein protein levels are determined by image analysis of two-dimensional gels.
4. The method of claim 1, wherein the level of modification of ferritin light chain fragment, annexin A6, and myosin-IXa fragment are measured.
5. The method of claim 1 or 4, further comprising measuring the modification of parathyroid hormone receptor and/or  $\beta$  actin.
6. The method of claim 4 or 5, wherein the level of modification is measured by saturation fluorescence labeling.
7. A method of assessing a subject having Chagas disease comprising: measuring a level of cysteinyl-S-nitrosylation (SNO) of one or more of beta actin, annexin A6, ferritin light chain fragment (~18kDa), parathyroid hormone 2 receptor fragment (~20kDa), or myosin-IXa fragment; wherein elevated levels of nitrosylation is indicative of risk for developing chagasic cardiomyopathy.
8. The method of claim 7, further comprising measuring the modification of parathyroid hormone receptor and/or  $\beta$  actin.
9. The method of claim 7, further comprising measuring levels of one or more proteins selected from vimentin, gamma actin, or keratin 10.

10. The method of claim 9, wherein protein levels are determined by image analysis of two-dimensional gels.
11. The method of claim 7, wherein the level of modification is measured by saturation fluorescence labeling.
12. A computer implemented method for performing the method of claim 1 or 7.
13. A computer implemented method comprising the steps of (a) obtaining protein level measurements of one or more of vimentin, gamma actin, or keratin 10, and/or protein modification measurements of one or more of beta actin, annexin A6, ferritin light chain fragment (~18kDa), parathyroid hormone 2 receptor fragment (~20kDa), or myosin-IXa fragment, (b) transforming the obtained measurements to a score or ratio, and (c) determining if the measurements indicate the presence of or the risk of developing cardiomyopathy.
14. A method of treating a patient at risk of chagasic cardiomyopathy comprising: administering a treatment for cardiomyopathy to a patient having elevated levels of (i) one or more proteins selected from vimentin, gamma actin, or keratin 10, or (ii); level of cysteinyl-S-nitrosylation (SNO) of one or more of beta actin, annexin A6, ferritin light chain fragment (~18kDa), parathyroid hormone 2 receptor fragment (~20kDa), or myosin-IXa fragment.
15. The method of claim 14, wherein the patient is an asymptomatic patient.
16. The method of assessing a subject with a trypanosome infection comprising measuring the level of one or more protein listed in table III.

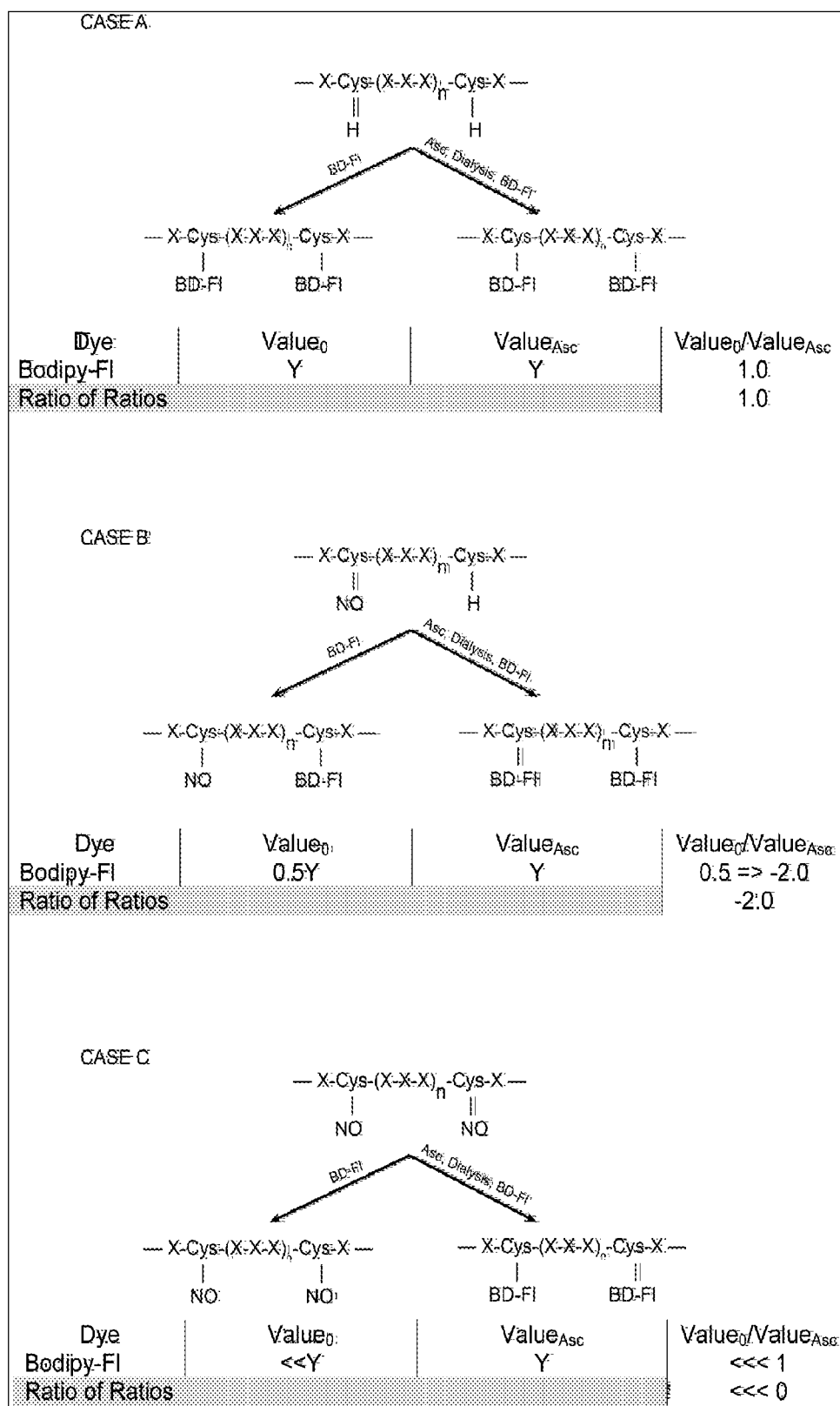


FIG. 1

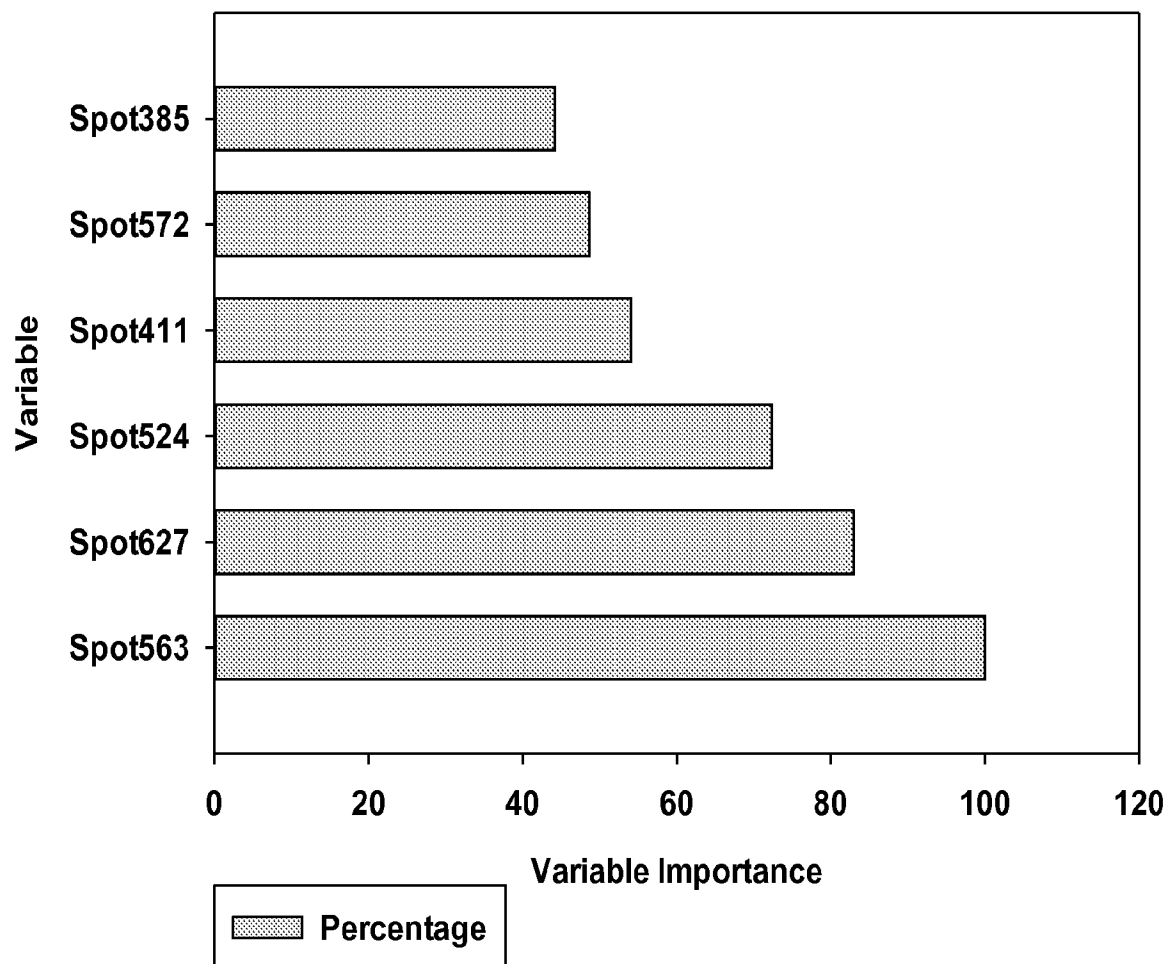


FIG. 2

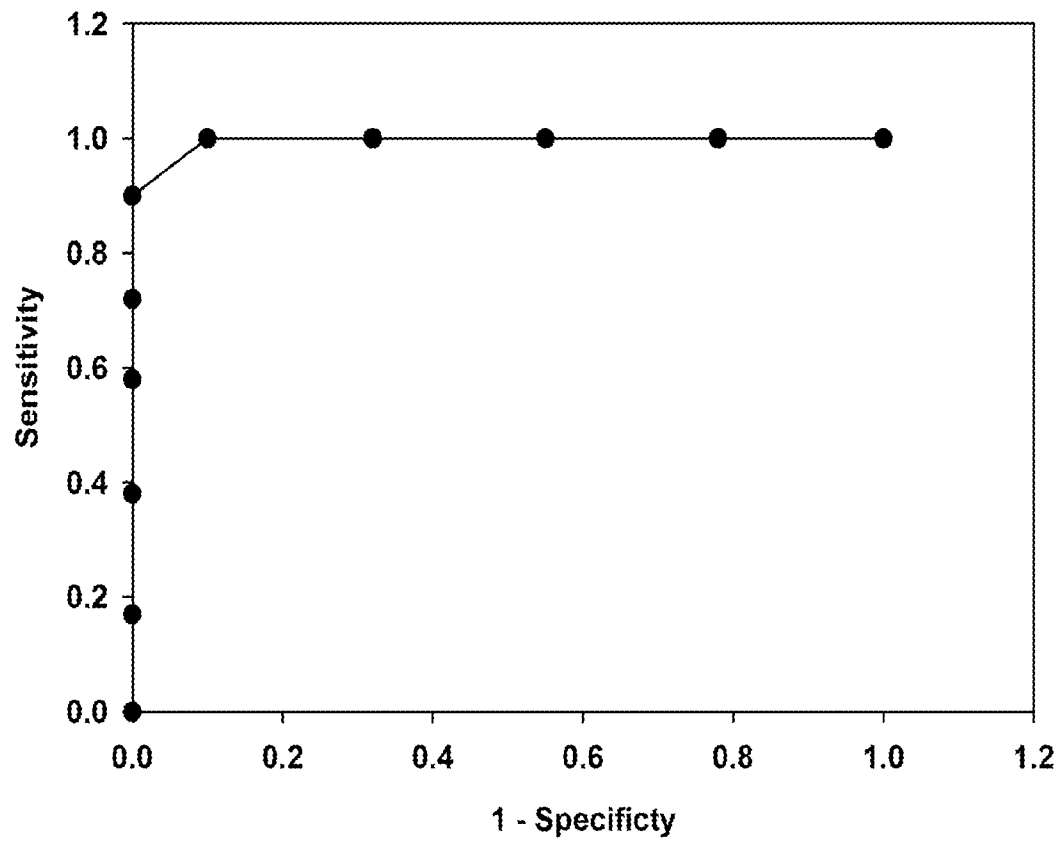


FIG. 3

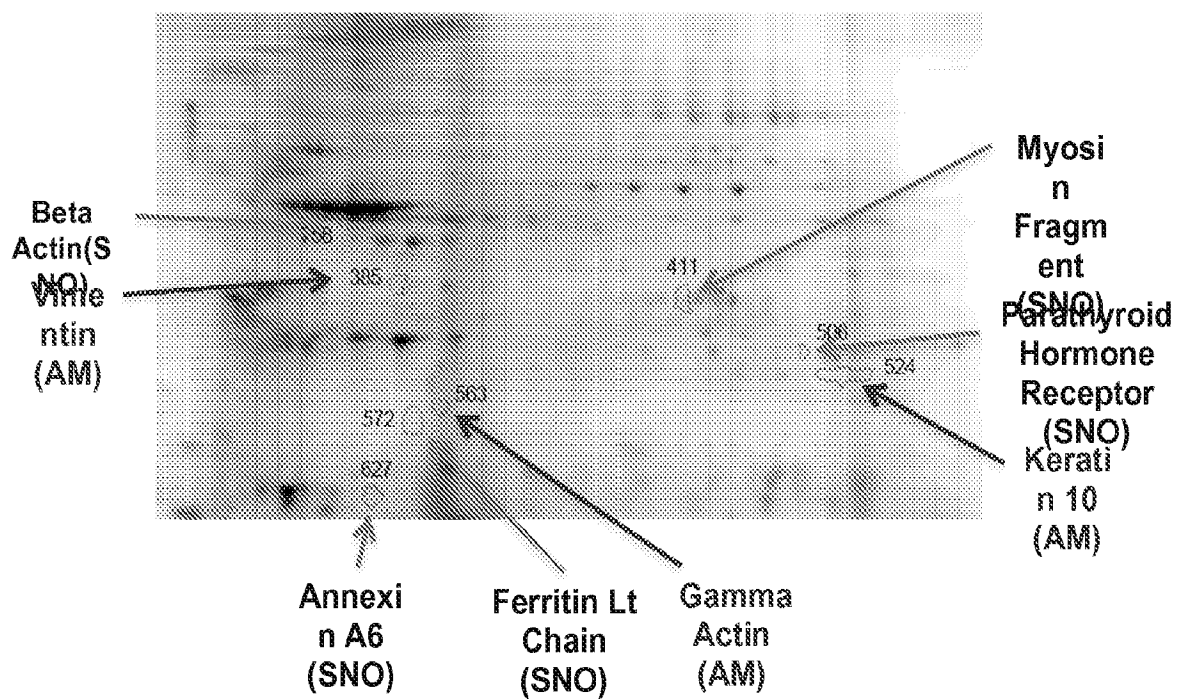


FIG. 4

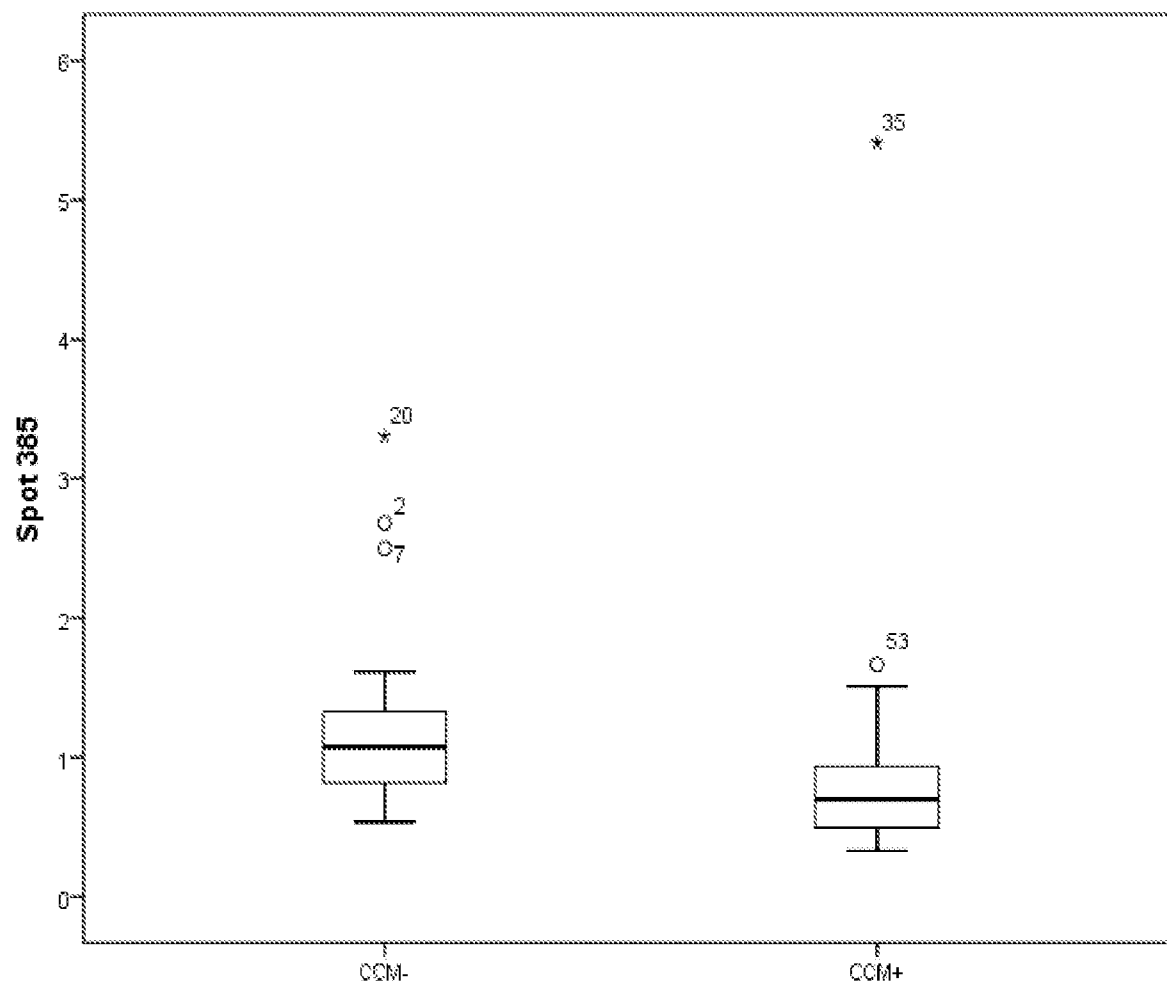


FIG. 5

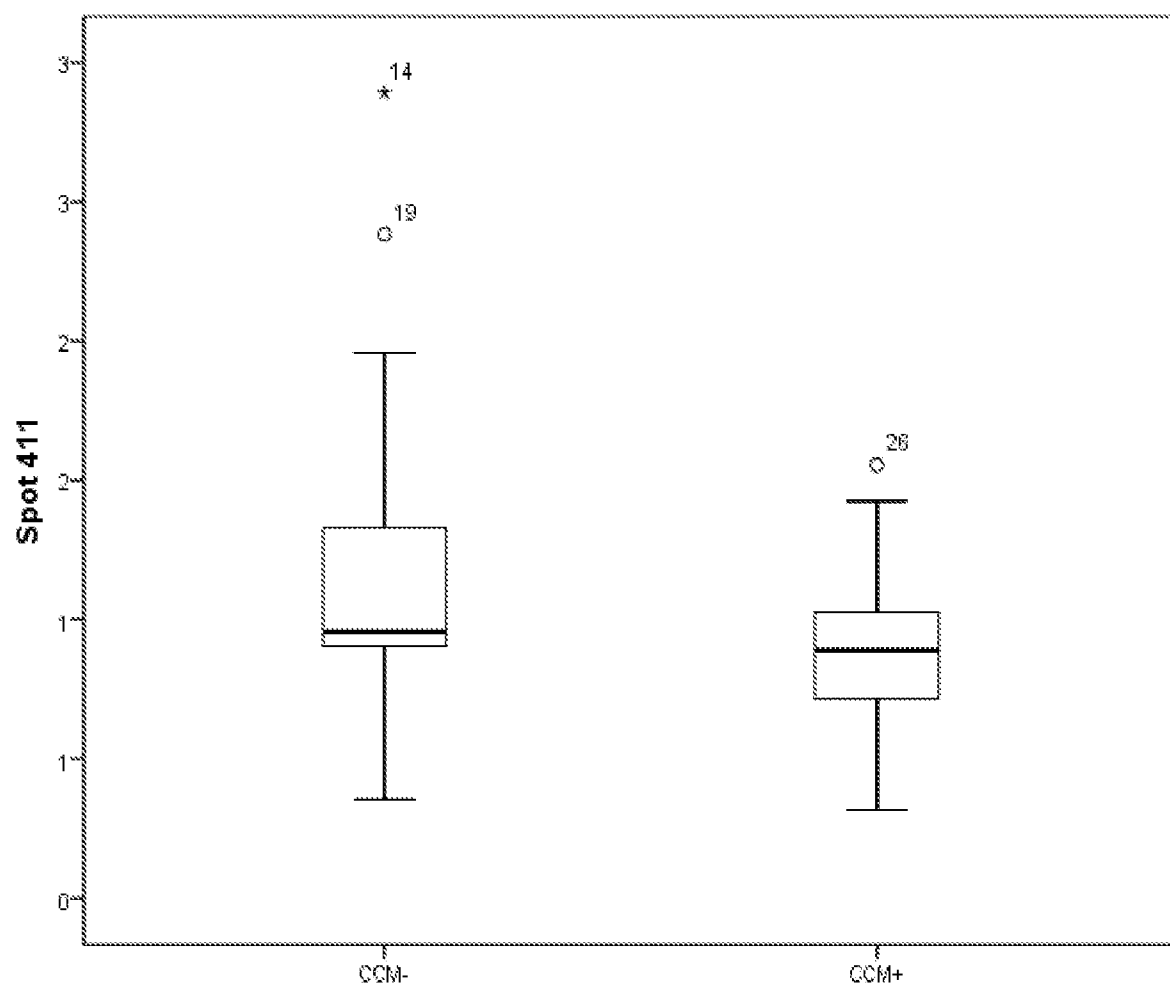


FIG. 6

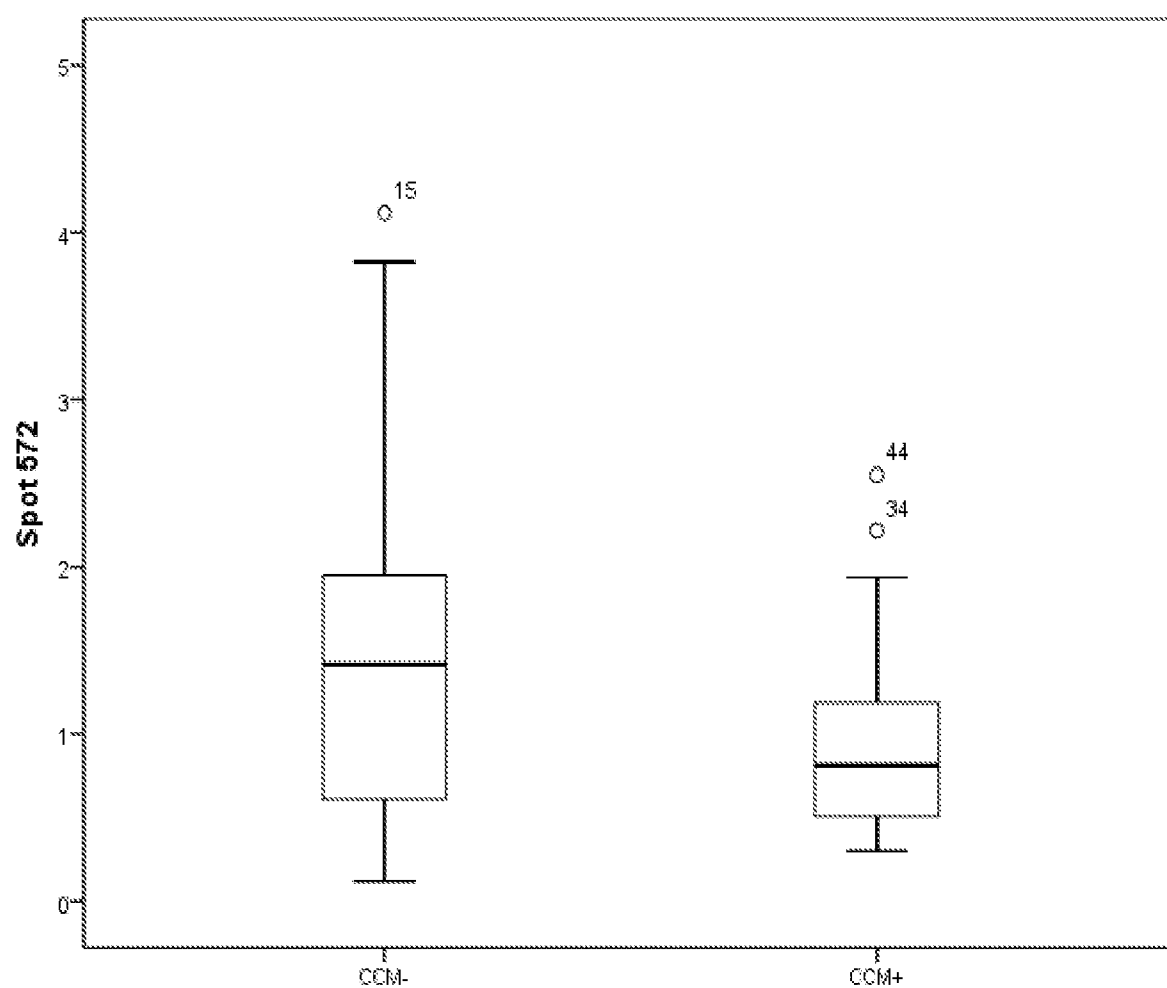


FIG. 7

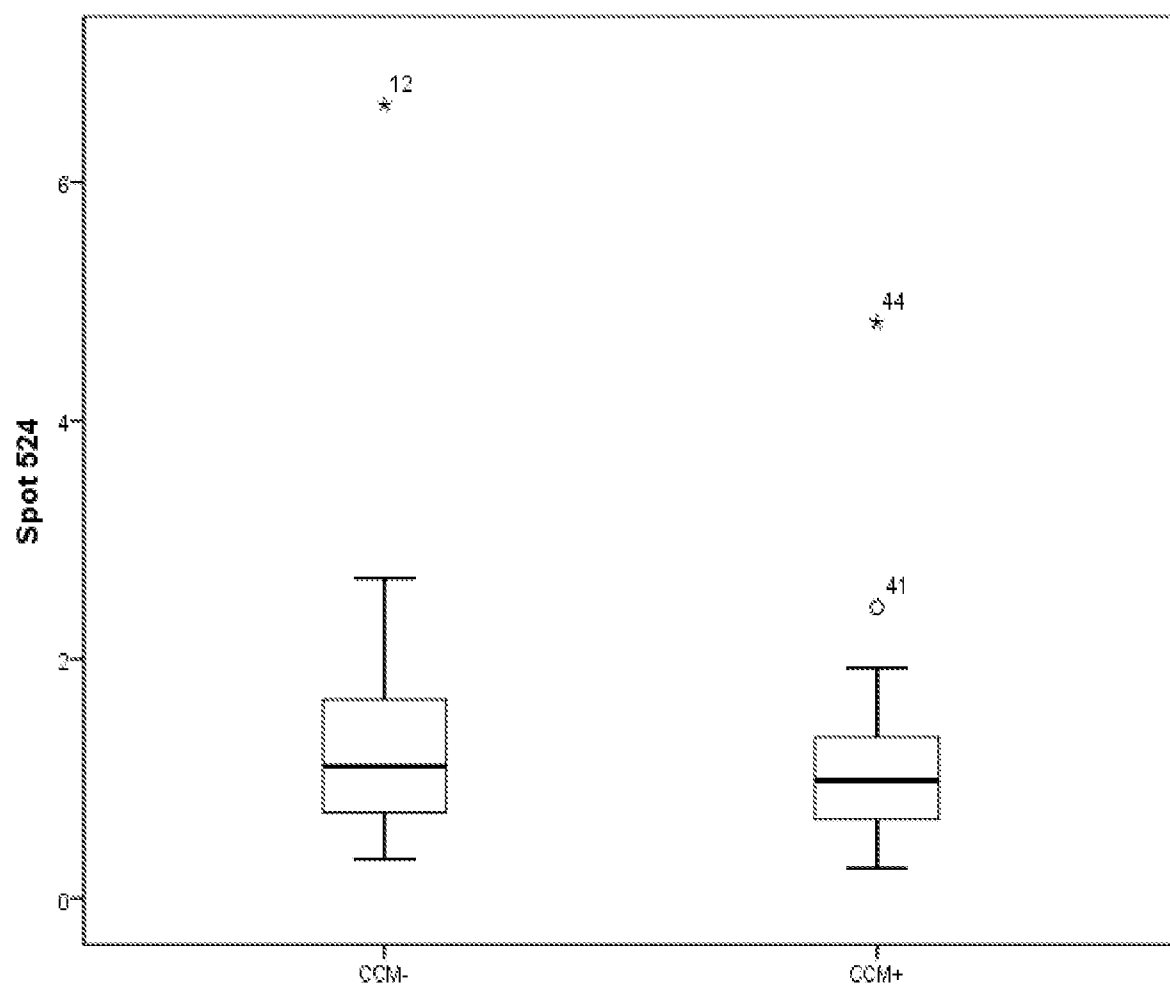


FIG. 8

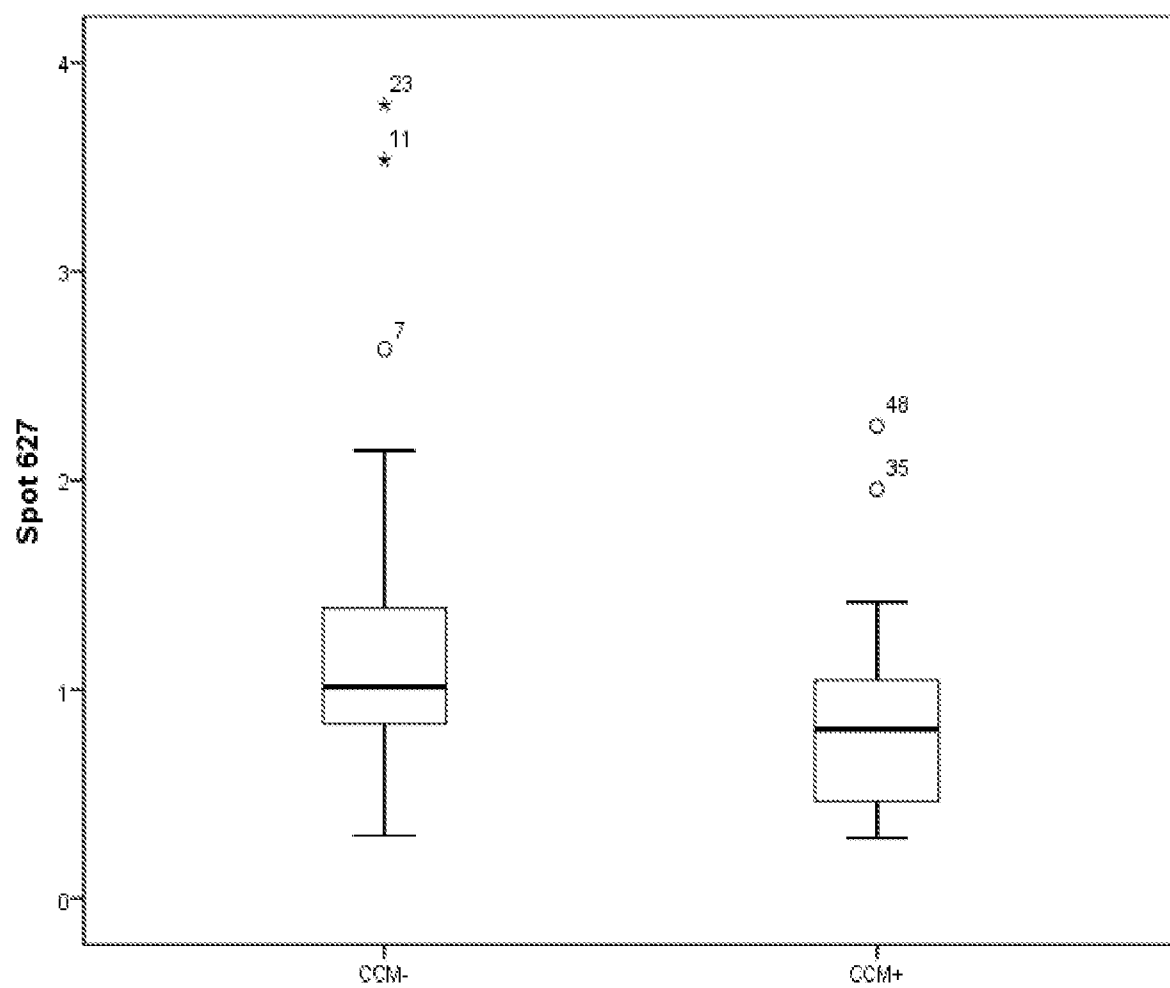


FIG. 9

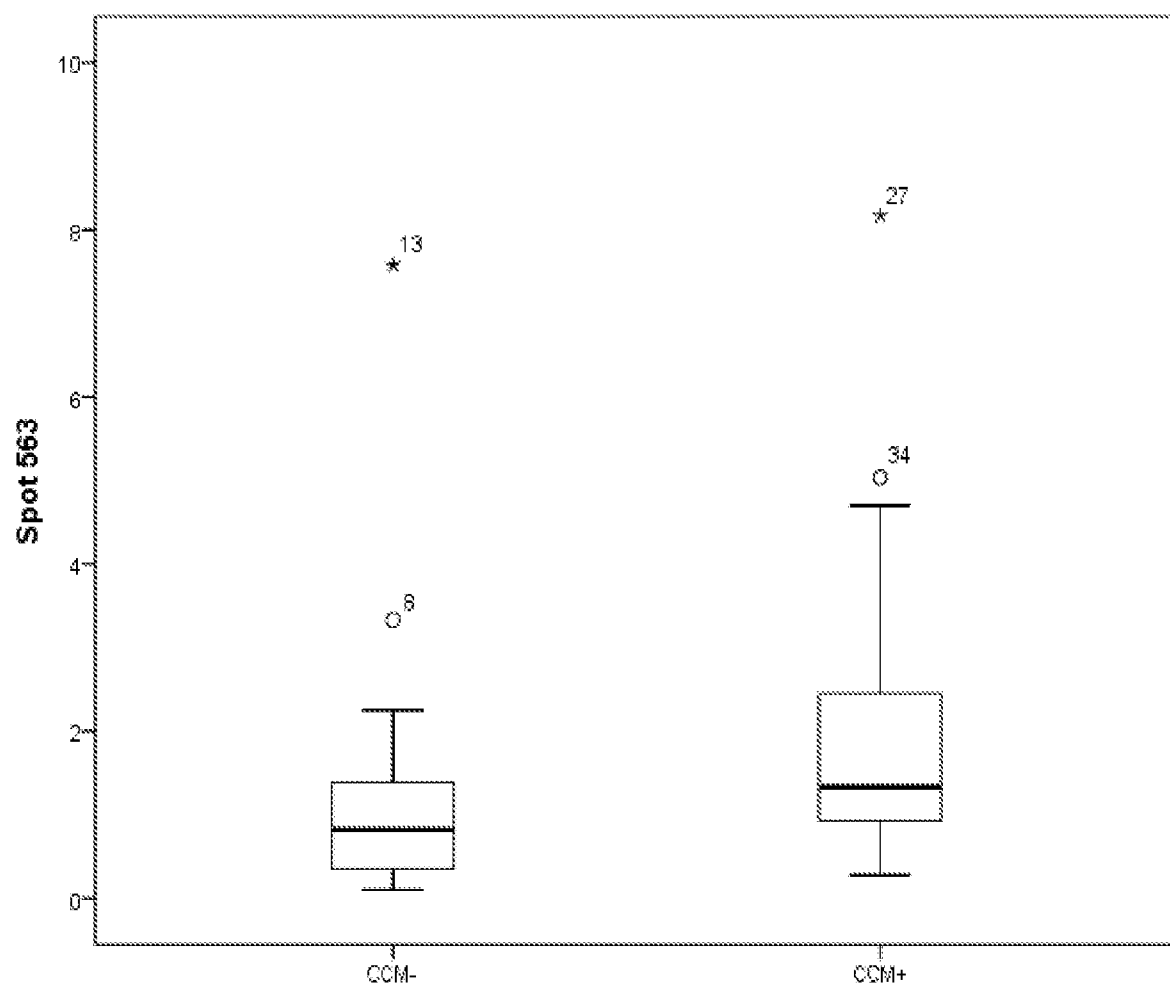


FIG. 10

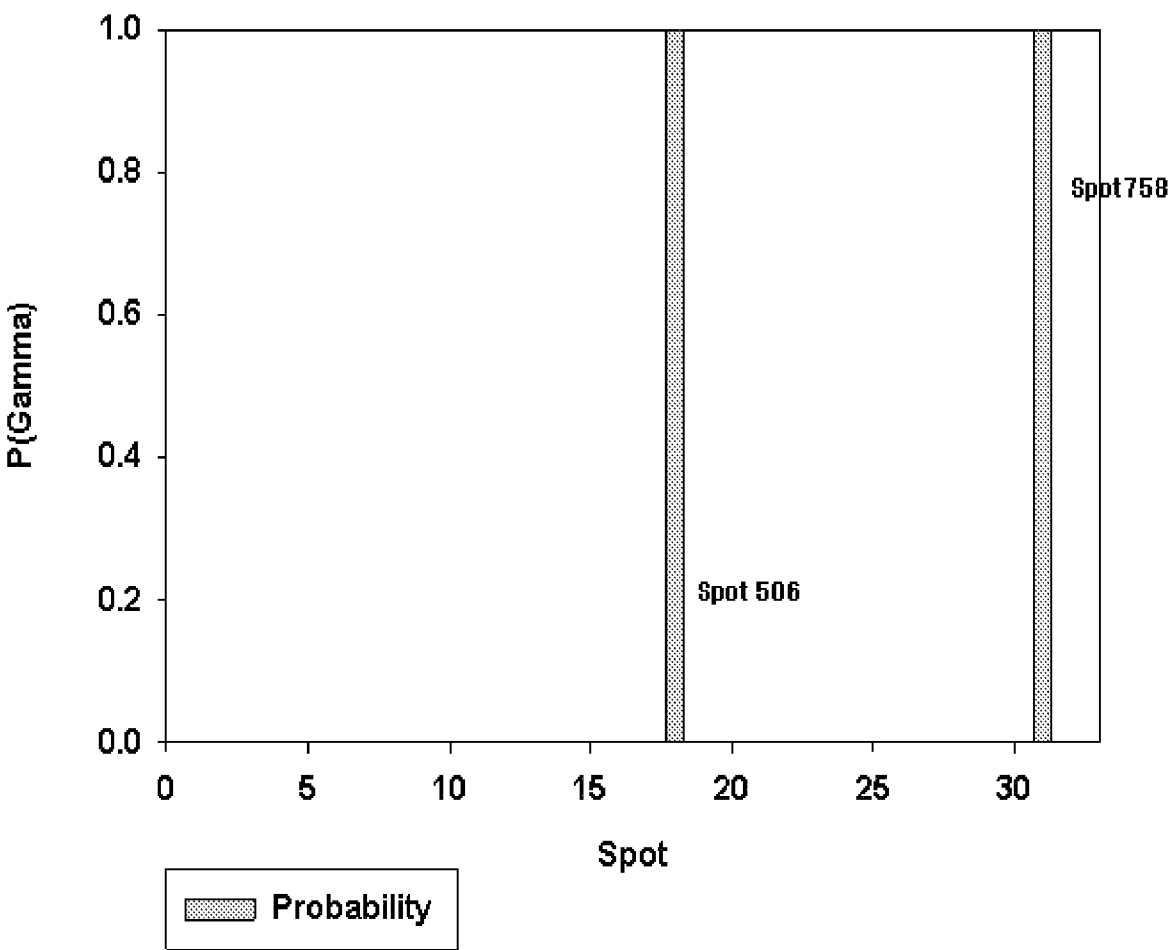


FIG. 11

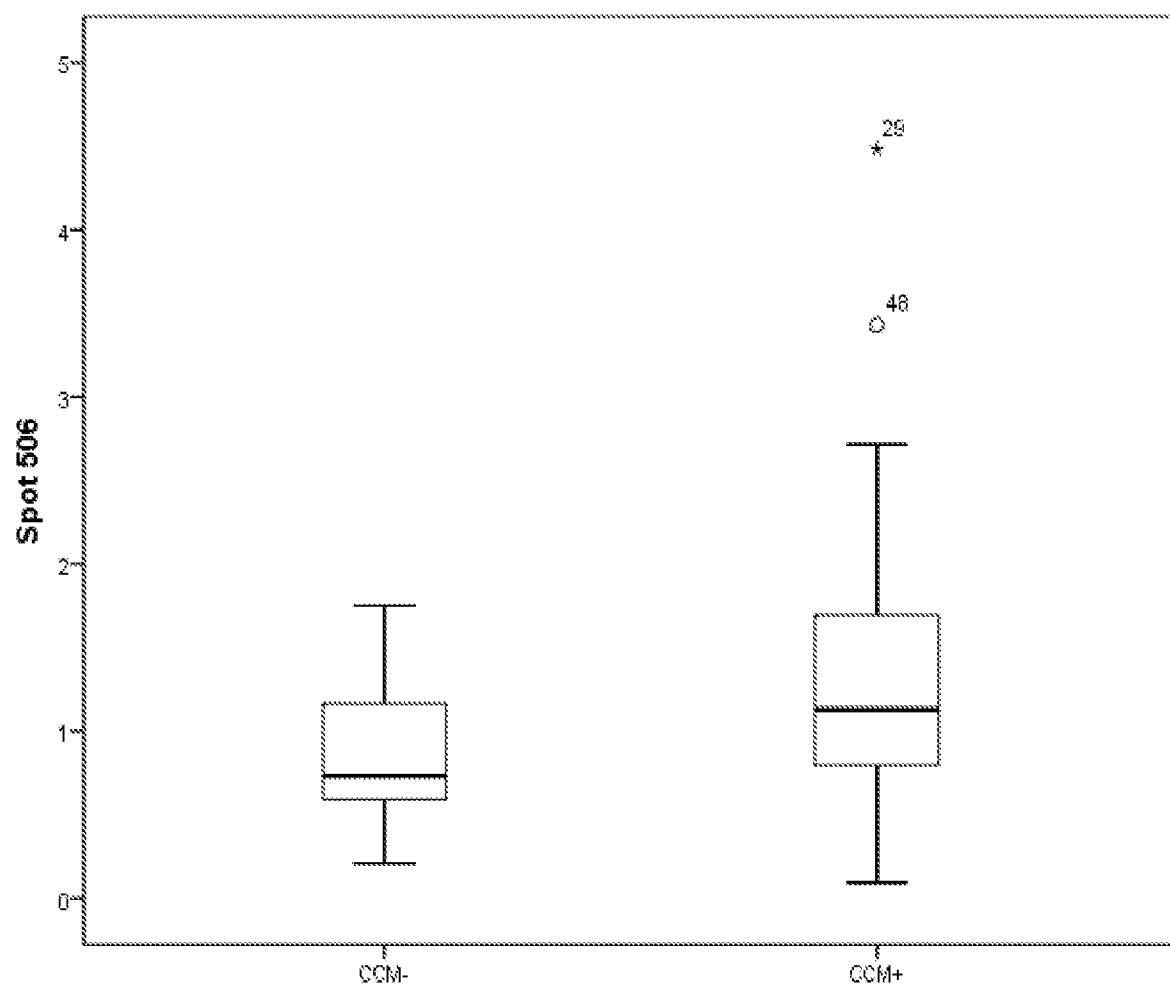


FIG. 12

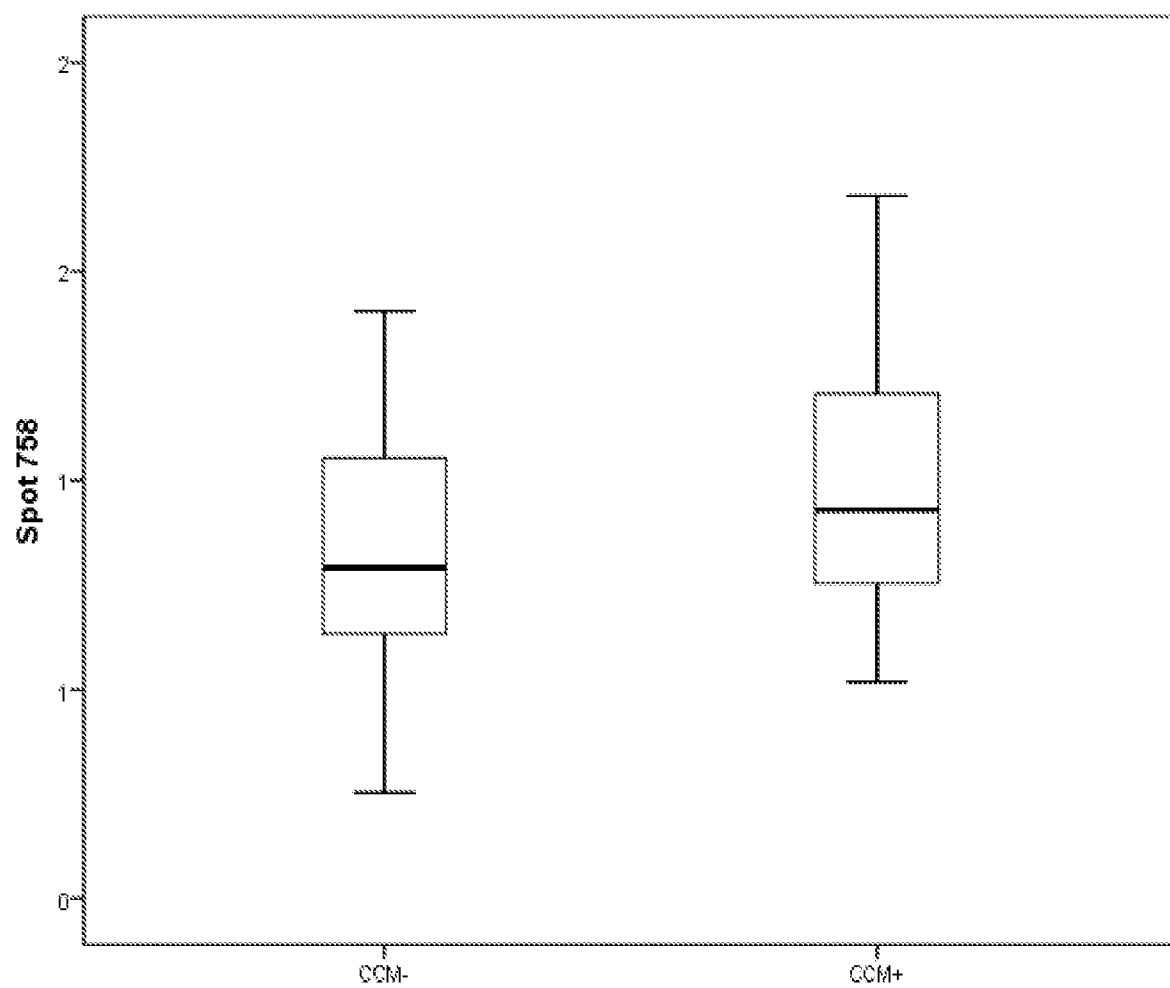


FIG. 13

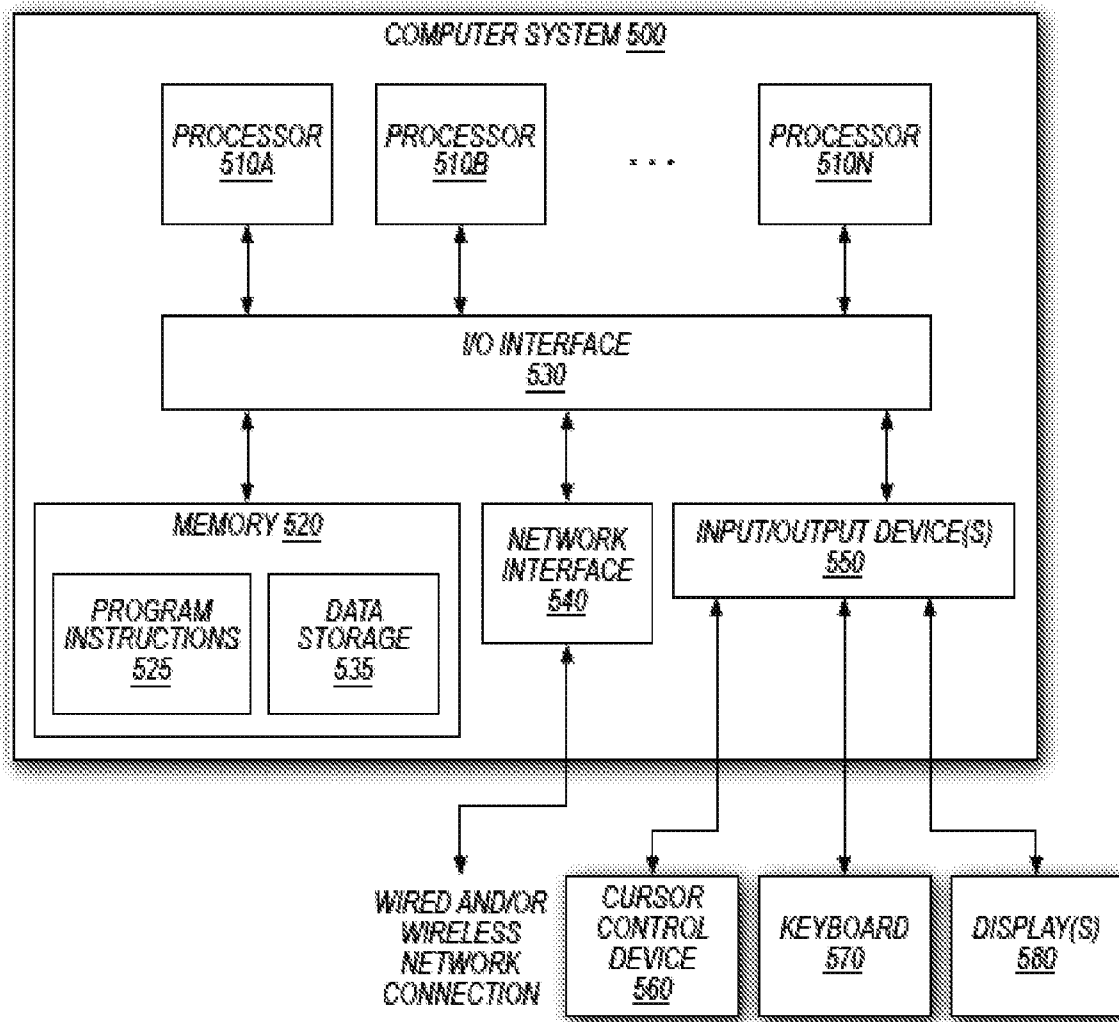


FIG. 14

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 14/22510

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12Q 1/68, G01N 33/53, G01N 33/00 (2014.01)

USPC - 435/6.17, 435/6.19, 435/7.1, 435/7.92

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8): C12Q 1/68, G01N 33/53, G01N 33/00 (2014.01)

USPC: 435/6.17, 435/6.19, 435/7.1, 435/7.92

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
CDC: C12Q 1/6883, A61K 38/00, G01N 33/564

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PatBase, PubWest, Google Scholar, Google Patents: Chagas disease, chagas cardiomyopathy, vimentin, nitrosylation, gamma actin, AGTG1, keratin 10, CK-10, K10, two-dimensional, 2DGE, ferritin light chain fragment, ferritin, annexin A6, ANXA6, myosin-lxa, MYO9A, parathyroid hormone receptor or PTH, p actin, computer

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category*       | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                       | Relevant to claim No.          |
|-----------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------|
| X<br>-----<br>Y | US 2012/0316211 A1 (GARG) 13 December 2012 (13.12.2012); para [0009], [0010], [0011], [0012], [0058], [0073]                                                                                                                             | 1, 14-16<br>-----<br>2-5, 7-13 |
| Y               | WO 2011/127219 A1 (KUSLICH et al.) 13 October 2011 (13.10.2011); abstract; para [0010], [0012], [0023], [0173]                                                                                                                           | 2-4, 5/4                       |
| Y               | US 2011/0195425 A1 (SELINFREUND et al.) 11 August 2011 (11.08.2011); abstract; para [0072]                                                                                                                                               | 5/1, 8, 5/4                    |
| Y               | ALMEIDA et al., Subcellular Proteomics and Global Analysis of Posttranslational Modifications to Study Functional Roles of Trypanosoma cruzi Molecules, The Open Parasitology Journal, 2010, Vol. 4, pages 167-177; p.174, col 2, para 2 | 7-11, 12/7                     |
| Y               | US 6,535,624 B1 (TAYLOR) 18 March 2003 (18.03.2003); abstract                                                                                                                                                                            | 12-13                          |
| Y               | US 2002/0091248 A1 (ADAMS et al.) 11 July 2002 (11.07.2002); abstract                                                                                                                                                                    | 4, 5/4                         |
| Y               | PRETZER et al., Saturation fluorescence labeling of proteins for proteomic analyses, Analytical Biochemistry, 8 August 2007, Vol. 374, pages 250?262; abstract; p.250, col 2, para 2                                                     | 11                             |

☐ Further documents are listed in the continuation of Box C.

\* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

9 August 2014 (09.08.2014)

Date of mailing of the international search report

09 SEP 2014

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents  
P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-3201

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PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 14/22510

## Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 6  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.