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(71) Applicant *(for all designated States except US)*: **ABBOTT LABORATORIES** [US/US]; 100 Abbott Park Road, Abbott Park, Illinois 60064 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **MATTINGLY, Phillip, G.** [US/US]; 204 Seafarer Drive, Third Lake, Illinois 60030-2640 (US). **ADAMCZYK, Maciej, B.** [US/US]; 174 Quail Haven Court, Gurnee, Illinois 60031 (US). **BRASHEAR, Roy Jeffrey** [US/US]; 145 N. Sylvan Drive, Mundelein, Illinois 60060-3307 (US). **DOSS, Robert, C.** [US/US]; 1300 Canterbury Circle, Libertyville, Illinois 60048 (US).

(74) Agent: **BARTNICKI, Audrey, L.**; Abbott Laboratories, Dept. 0377, Bldg. AP6A-1A, 100 Abbott Park Road, Abbott Park, Illinois 60064-6008 (US).

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(54) Title: CARDIOVASCULAR AUTOIMMUNE DISEASE PANEL AND METHODS OF USING SAME

(57) Abstract: Provided herein among other things are diagnostic tests, methods of use, and kits for the assessment and management of cardiovascular autoimmune disease and risk of cardiovascular autoimmune disease. Assay methods of the invention can be employed among other things to identify cardiovascular autoimmune disease, or risk thereof, in subjects who have cardiovascular disease, an autoimmune disease, or who are related to an individual with an autoimmune disease. The method can be employed for testing of a subject that exhibits symptoms of cardiovascular disease, as well as of a subject that is apparently healthy and does not yet exhibit symptoms of cardiovascular disease, but may with time. In one embodiment, the invention also provides a method of determining whether a subject having, or at risk for, a cardiovascular disease is a candidate for immunosuppressive therapy or immunoabsorption therapy. The invention also provides kits and kit components that are useful for performing the methods of the invention.

CARDIOVASCULAR AUTOIMMUNE DISEASE PANEL AND METHODS OF USING SAME

TECHNICAL FIELD

The present invention relates generally to the diagnosis, management and therapy of cardiovascular autoimmune disease. In particular, the invention relates among other things to diagnostic tests, methods of use, and kits related to the assessment and management of cardiovascular autoimmune disease and risk of cardiovascular autoimmune disease. In one embodiment, the invention provides methods and means for risk stratification of cardiovascular autoimmune disease.

BACKGROUND

Autoimmune disease has been implicated in over 80 serious illnesses that involve almost every human organ system. The increased incidence of cardiovascular pathologies in patients with established autoimmune disease has been reported in the scientific literature (e.g., systemic lupus erythematosus, rheumatoid arthritis, thyroiditis, and others) (Frostegard, Arterioscler. Thromb. Vasc. Biol., 15:1776-1785 (2005)). Autoantibodies to many endogenous antigens, including those indicative of cardiovascular pathologies, have been detected. Autoantibodies to oxidized LDL can be detected in patients with normal and abnormal coronary angiograms, but titers are significantly higher in patients with angiographic evidence of coronary artery disease than in normal subjects and patients with normal coronary angiograms (Bui et al., American Heart Journal, 131 (4), 663-667 (1996)). An autoimmune basis likewise has been suggested for myocarditis and dilated cardiomyopathy (Maisch, Herz, 30, 535-544 (2005); Caforio et al., G. Ital. Cardiol., 27, 106-112 (1997)).

Autoantibodies also have been observed to contribute to the generation of false negative results obtained in cardiac biomarker studies including troponin assays (e.g., Bohner et al., Clin. Chem., 42, 2046 (1996)). This, and additional recent evidence, supports the novel conclusion underlying the subject invention that the onset of cardiovascular disease (CD) likely involves an autoimmune component which starts years or decades earlier before clinical manifestations. A patient may have heart disease without experiencing pain, thus delaying any visit to the physician and any diagnosis.

CD is one of the leading causes of death in the United States. CD is a clinical syndrome that is progressive, may develop slowly, and may be chronic once established. The importance of early detection of cardiovascular disease of an autoimmune nature -- cardiovascular autoimmune disease (also known as CAD) -- cannot be overstated. Early detection of cardiovascular autoimmune disease provides a greater opportunity for the initiation of treatment and the potential for recovery, especially in patients that are non-responsive to conventional therapy and/or in whom immunosuppressives would be of benefit. Moreover, the treatment method of choice necessarily may differ from that of standard CD treatment if it is determined early that the disease is of an autoimmune nature.

There are many biomarkers indicative of various cardiovascular pathologies that can be individually measured (e.g., troponin, brain natriuretic peptide (BNP), nt-proBNP, creatine kinase isoenzyme MB (CKMB), myoglobin, myeloperoxidase (MPO), choline, C-reactive protein (CRP), interleukin-6 (IL-6), tumor necrosis factor α (TNFα), placenta growth factor (P*l*GF), Pregnancy-Associated Plasma Protein-A (PAPP-A), soluble CD40 (sCD40), and others). Especially important are those markers employed for diagnosing acute coronary syndrome (ACS) to justify emergency intervention and hospitalization. Still, using these biomarkers a certain percentage of patients are not diagnosed correctly as having ACS, and consequently experience adverse outcomes. Part of the problem could stem from the fact that these endogenous cardiovascular antigens are often unstable in patient samples, and are subject to proteolysis or oxidation or other reactions, which complicates sample handling and limits any ability to re-analyze patient samples. Furthermore, these markers do not allow for defining risk in advance and preventing the development of cardiovascular physiopathologies.

Thus, clearly current diagnostic tools are inadequate for the detection of cardiovascular autoimmune disease. Cardiovascular disease is underdiagnosed, and is misdiagnosed, by general practitioners and experts alike. Tools are needed with which the risk of cardiovascular disease can be assessed.

Based on the foregoing, there remains a need for methods and means to assess risk of cardiovascular autoimmune disease that are independent of the biomarkers associated with acute manifestation of the disease. Therefore, it is an object of the

invention to provide among other things diagnostic tests, a panel of diagnostic tests, methods of use, and kits for the assessment of risk associated with cardiovascular disease. Autoantibodies have long circulating lifetimes in vivo and prolonged stability in vitro, and are much more robust analytes than are certain antigens. Thus, optimally the tests, test panels, methods and kits of the invention involve detection of autoantibodies to endogenous cardiovascular antigens, and thereby optionally avoid some of the pitfalls in cardiovascular disease testing which are inherent in the currently used methodologies. These and other objects will be apparent from the description provided herein.

The foregoing discussion of background information is provided merely to assist the reader in understanding the invention and is not admitted to describe or constitute prior art to the invention.

SUMMARY

The present invention relates generally to the diagnosis, management and therapy of cardiovascular autoimmune disease. In particular, the invention provides among other things diagnostic tests, methods of use, and kits related to the assessment and management of cardiovascular autoimmune disease and risk of cardiovascular autoimmune disease. The invention also provides methods and means for risk stratification of cardiovascular autoimmune disease.

In one embodiment, the present invention relates to a method for assessing the risk of whether a subject has (e.g., currently has) or might develop (e.g., at some point in the future) a cardiovascular autoimmune disease, wherein the method comprises:

(a) obtaining a sample from the subject;

(b) measuring the levels (i.e., amounts) of autoantibodies to each of a plurality of different endogenous cardiovascular antigens in the subject's sample;

(c) comparing the levels with the level of the same autoantibodies measured in samples from reference subjects with clinically normal cardiovascular function, or from reference subjects having cardiovascular disease, and

(d) identifying the risk that the subject has or might develop a cardiovascular autoimmune disease based on the comparison in step (c).

According to this method, such a risk is present when levels of autoantibodies to at least two of the different endogenous cardiovascular antigens:

(i) are elevated in the subject's sample as compared to in samples from reference subjects with clinically normal cardiovascular function, and/or

(ii) are elevated in or at about the same level in the subject's sample as compared to in samples from reference subjects having cardiovascular disease.

In one aspect, the risk is further increased when the subject either has an autoimmune disease, or is a first-degree relative of an individual having an autoimmune disease. In another aspect, the levels of autoantibodies to at least two of the different endogenous cardiovascular antigens are elevated due to the presence of cardiovascular autoimmune disease. In yet another aspect, the levels of autoantibodies to at least two of the different endogenous cardiovascular antigens are elevated due to an increased risk of developing cardiovascular autoimmune disease.

The subject's sample used in the above method can comprise peripheral blood, serum, plasma, cerebrospinal fluid, urine, or other body fluid sample.

In the above method, the cardiovascular autoimmune disease is a disease, disorder or condition selected from the group consisting of myocarditis, cardiomyopathy, and ischemic heart disease.

Optionally, the "plurality" of different endogenous cardiovascular antigens in the subject's sample comprises two or more endogenous cardiovascular antigens. Further optionally, wherein the plurality comprises between two and ten, especially between five and ten. In particular, optionally the plurality comprises a number selected from the group consisting of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 15, 17, 18, 19, and 20.

The different endogenous cardiovascular antigens employed in the method are selected from the group consisting of α1-adrenoceptor, angiotensin-1 receptor, annexin V, brain natriuretic peptide, cardiac troponins, myosin, tropomyosin, cytoplasmic neutrophils, endothelial receptor of protein C, Factor VIII, grehlin, halogenated protein, nitrated protein, heat shock proteins, myeloperoxidase, placental growth factor, phospholipids, proteinase-3, prothrombin, Purkinje fibers, sarcolemmal Na-K-ATPase, β1-adrenoceptor, β2-adrenoceptor, and tissue-type plasminogen activator. In another aspect, the different endogenous cardiovascular antigens employed in the method

further comprise an antigen selected from the group consisting of cardiolipin, oxidized LDL, and β-2-glycoprotein-1.

In another embodiment, the present invention relates to a method for identifying a patient with cardiovascular disease as eligible to receive autoimmune therapy, wherein the method comprises:

(a) obtaining a sample from the cardiovascular disease patient;

(b) measuring the levels of autoantibodies to each of a plurality of different endogenous cardiovascular antigens in the subject's sample;

(c) comparing the levels with the level of the same autoantibodies measured in samples from reference subjects with clinically normal cardiovascular function, or from reference subjects having autoimmune disease; and

(d) identifying the cardiovascular disease patient as eligible to receive autoimmune therapy based on the comparison in step (c).

According to such a method, the patient is eligible to receive autoimmune therapy when levels of autoantibodies to at least two of the different endogenous cardiovascular antigens:

(i) are elevated in the cardiovascular disease patient's sample as compared to in samples from reference subjects with clinically normal cardiovascular function, and/or

(ii) are elevated in or at about the same in the cardiovascular disease patient's sample as compared to in samples from reference subjects having autoimmune disease.

Additionally, according to such a method, the cardiovascular disease patient further is eligible to receive autoimmune therapy when the subject either has an autoimmune disease, or is a first-degree relative of an individual having an autoimmune disease.

In one aspect of this method, the levels of autoantibodies to at least two of the different endogenous cardiovascular antigens are elevated due to the presence of cardiovascular autoimmune disease. In another aspect of this method, the levels of autoantibodies to at least two of the different endogenous cardiovascular antigens are elevated due to an increased risk of developing cardiovascular autoimmune disease.

The subject's sample used in the above method can comprise peripheral blood, serum, plasma, cerebrospinal fluid, urine, or other body fluid sample.

In the above method, the cardiovascular autoimmune disease is a disease, disorder or condition selected from the group consisting of myocarditis, cardiomyopathy, and ischemic heart disease.

Optionally, the "plurality" of different endogenous cardiovascular antigens in the subject's sample comprises two or more endogenous cardiovascular antigens. Further optionally, wherein the plurality comprises between two and ten, especially between five and ten. In particular, optionally the plurality comprises a number selected from the group consisting of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 15, 17, 18, 19, and 20.

The different endogenous cardiovascular antigens employed in the method are selected from the group consisting of α1-adrenoceptor, angiotensin-1 receptor, annexin V, brain natriuretic peptide, cardiac troponins, myosin, tropomyosin, cytoplasmic neutrophils, endothelial receptor of protein C, Factor VIII, grehlin, halogenated protein, nitrated protein, heat shock proteins, myeloperoxidase, placental growth factor, phospholipids, proteinase-3, prothrombin, Purkinje fibers, sarcolemmal Na-K-ATPase, β1-adrenoceptor, β2-adrenoceptor, and tissue-type plasminogen activator. In another aspect, the different endogenous cardiovascular antigens employed in the method further comprise an antigen selected from the group consisting of cardiolipin, oxidized LDL, and β-2-glycoprotein-1.

In still a further embodiment, the invention provides a method of identifying a subject having, or at risk for developing, a cardiovascular autoimmune disease, the method comprising assaying a sample from the subject for the levels of autoantibodies to each of a plurality of different endogenous cardiovascular antigens, wherein the subject has an autoimmune disease, or the subject is a first-degree relative of an individual having an autoimmune disease, and wherein the presence of elevated levels of autoantibodies to at least two of the different endogenous cardiovascular antigens indicates the presence of, or risk of, the cardiovascular autoimmune disease.

According to the invention, also provided herein is a test kit for assaying a sample for autoantibodies to at least two different endogenous cardiovascular antigens, the test kit comprising means for the detection of levels of autoantibodies to each of a plurality of different endogenous cardiovascular antigens. In one aspect, the test kit additionally may comprise a solid phase and a capture agent affixed to the solid phase,

wherein the capture agent is a peptide having a sequence that corresponds to a portion or the entirety of the amino acid sequence of the endogenous cardiovascular antigens selected from the group consisting of α1-adrenoceptor, angiotensin-1 receptor, annexin V, brain natriuretic peptide, cardiac troponins, myosin, tropomyosin, cytoplasmic neutrophils, endothelial receptor of protein C, Factor VIII, grehlin, halogenated protein, nitrated protein, heat shock proteins, myeloperoxidase, placental growth factor, phospholipids, proteinase-3, prothrombin, Purkinje fibers, sarcolemmal Na-K-ATPase, β1-adrenoceptor, β2-adrenoceptor, and tissue-type plasminogen activator. In another aspect, the different endogenous cardiovascular antigens may further comprise an antigen selected from the group consisting of cardiolipin, oxidized LDL, and β-2-glycoprotein-1.

The test kit additionally optionally comprises a labeled detection agent, comprising a species-specific antibody, and further optionally comprises an indicator reagent that interacts with the label to produce a detectable signal.

In one aspect, the solid phase of the test kit comprises a microplate

In another aspect, the solid phase of the test kit comprises a microparticle.

In still a further aspect, the solid phase of the test kit comprises an electrode.

In an additional embodiment, the present invention relates to a method for assessing the risk of whether a subject has or might develop a cardiovascular autoimmune disease, wherein the method comprises:

(a) obtaining a sample from the subject;

(b) measuring the levels of one or more endogenous cardiovascular antigens in the subject's sample;

(c) measuring the levels of autoantibodies to each of a plurality of different endogenous cardiovascular antigens in the subject's sample;

(d) comparing the levels of the one or more endogenous cardiovascular antigens with the level of the same endogenous cardiovascular antigen measured in samples from reference subjects with clinically normal cardiovascular function, in samples from reference subjects having cardiovascular disease, or in the subject's sample obtained at an earlier time;

(e) comparing the levels of autoantibodies with the level of the same autoantibody measured in samples from reference subjects with clinically normal

cardiovascular function, in samples from reference subjects having cardiovascular disease, or in the subject's sample obtained at an earlier time; and

(f) identifying the risk that the subject has or might develop a cardiovascular autoimmune disease based on the comparison in steps (d) and (e).

Optionally, the measuring of step (b) and the measuring of step (c) are done simultaneously. Further optionally, the measuring of step (b) and the measuring of step (c) are done sequentially, in any order.

In one aspect of such a method as described herein, the risk is present when:

(i) in step (d) the levels of the one or more endogenous cardiovascular antigens are altered (e.g., elevated in) in the subject's sample as compared to in samples from reference subjects with clinically normal cardiovascular function, are altered in (e.g., elevated in) or at about the same level in the subject's sample as compared to in samples from reference subjects having cardiovascular disease, and/or are altered in (e.g., elevated in) or at about the same level in the subject's sample as compared to in the subject's sample obtained at an earlier time; and/or

(ii) in step (e) the levels of the autoantibodies to at least two of the different endogenous cardiovascular antigens are altered in (e.g., elevated in) the subject's sample as compared to in samples from reference subjects with clinically normal cardiovascular function, are altered in (e.g., elevated in) or at about the same level in the subject's sample as compared to in samples from reference subjects having cardiovascular disease, and/or are altered in (e.g., elevated in) or at about the same level in the subject's sample as compared to in the subject's sample obtained at an earlier time.

In another aspect of such a method as described herein, the risk is present when:

(i) in step (d) the levels of the one or more endogenous cardiovascular antigens are altered in the subject's sample as compared to in samples from reference subjects with clinically normal cardiovascular function, and/or are altered in or at about the same level in the subject's sample as compared to in the subject's sample obtained at an earlier time; and/or

(ii) in step (e) the levels of the autoantibodies to at least two of the different endogenous cardiovascular antigens are elevated in the subject's sample as compared to in samples from reference subjects with clinically normal cardiovascular function,

and/or are elevated in or at about the same level in the subject's sample as compared to in samples from reference subjects having cardiovascular disease.

These and other features, aspects, objects, and embodiments of the invention will become more apparent in the following detailed description which contains information on exemplary features, aspects, objects and embodiments of the invention.

DETAILED DESCRIPTION

Surprising and unexpectedly, it has been discovered that autoantibodies to endogenous cardiovascular antigens are found in apparently healthy individuals and are of pathological nature, leading to cardiac physiopathology. Such autoantibodies to endogenous cardiovascular antigens can be assessed using diagnostic tests, methods, and kits as described herein. Among other things, the levels of these autoantibodies (e.g., levels of autoantibodies to each of a plurality of different endogenous cardiovascular antigens) can be used to clinically evaluate subjects at risk of developing or currently suffering from cardiovascular autoimmune disease. The invention optionally provides a means of risk assessment that is independent of the biomarkers currently employed for the acute manifestation of cardiovascular disease. The data obtained using the diagnostic tests, methods, and kits as described herein also can be used to monitor the efficacy of a treatment regimen, and optionally can be employed in an emergency setting as well as in a non-emergency setting. Particularly advantageous, the method can be employed for testing of a subject that exhibits symptoms of cardiovascular disease, as well as of a subject that is apparently healthy and does not yet exhibit symptoms of cardiovascular disease, but may with time.

The present invention thus provides, among other things, diagnostic tests, methods of use, and kits for the assessment and management of cardiovascular autoimmune disease and risk of cardiovascular autoimmune disease. In one embodiment, the invention provides methods and means for risk stratification of cardiovascular autoimmune disease. These and additional embodiments, features, aspects, illustrations, and examples of the invention are further described in the sections which follow.

Definitions

Unless defined otherwise herein, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

The term "risk" relates to the possibility or probability of a particular event occurring either presently, or, at some point in the future. "Risk stratification" refers to an arraying of known clinical risk factors to allow physicians to classify patients into a low, moderate, high or highest risk of developing of a particular disease, disorder or condition.

"Diagnosing" according to the invention includes determining, monitoring, confirmation, subclassification, and prediction of the relevant disease, complication, or risk. "Determining" relates to becoming aware of a disease, complication, risk, or entity (e.g., autoantibody). "Monitoring" relates to keeping track of an already diagnosed disease, complication, or risk factor, e.g., to analyze the progression of the disease or the influence of a particular treatment on the progression of disease or complication. "Confirmation" relates to the strengthening or substantiating of a diagnosis already performed using other indicators or markers. "Classification" or "subclassification" relates to further defining a diagnosis according to different subclasses of the diagnosed disease, disorder or condition, e.g., defining according to mild, moderate, or severe forms of the disease or risk. "Prediction" relates to prognosing a disease, disorder, condition, or complication before other symptoms or markers have become evident or have become significantly altered.

The term "cardiovascular autoimmune disease" as used herein refers to any deviation from a healthy or normal condition of the heart that is due to an underlying autoimmune disease, including any structural or functional abnormality of the heart, or of the blood vessels supplying the heart, that impairs its normal functioning. Examples of cardiovascular autoimmune diseases include myocarditis, cardiomyopathy, and ischemic heart disease, each due to an underlying autoimmune disease.

"Autoimmune disease" refers to the loss of immunological tolerance to self antigens. Some criteria for a diagnosis of autoimmune disease include: (1) the presence of circulating autoantibodies; (2) autoantibodies observed in the affected organ; (3) target antigen identified; (4) inducible in an animal model either by

immunization with antigen, serum, or autoantibody transfer; and (5) responsive to immunosuppressive therapy or immunoabsorption. Other characteristics of autoimmune disease include its: (a) increased prevalence in women; (b) familial clustering (although this varies with disease); (c) asymptomatic risk (i.e., the presence of autoantibodies may precede the disease by years); (d) periodic nature; and (e) chronic nature.

"Autoimmunity" refers to one or more immune responses directed against host antigens, characterized, for example, by the presence of autoantibodies or T lymphocytes reactive with host antigens.

The term "myocarditis" refers to inflammation of the myocardium. Myocarditis can be caused by a variety of conditions such as viral infection, sarcoidosis, rheumatic fever, autoimmune diseases (such as systemic lupus erythematosus, etc.), and pregnancy.

The term "cardiomyopathy" refers to a weakening of the heart muscle or a change in heart muscle structure. It is often associated with inadequate heart pumping or other heart function abnormalities. Cardiomyopathy can be caused by viral infections, heart attacks, alcoholism, long-term, severe high blood pressure, nutritional deficiencies (particularly selenium, thiamine, and L-carnitine), systemic lupus erythematosus, celiac disease, and end-stage kidney disease. Types of cardiomyopathy include dilated cardiomyopathy, hypertrophic cardiomyopathy, and restrictive cardiomyopathy.

As used herein, the term "dilated cardiomyopathy" refers to a global, usually idiopathic, myocardial disorder characterized by a marked enlargement and inadequate function of the left ventricle. Dilated cardiomyopathy includes ischemic cardiomyopathy, idiopathic cardiomyopathy, hypertensive cardiomyopathy, infectious cardiomyopathy, alcoholic cardiomyopathy, toxic cardiomyopathy, and peripartum cardiomyopathy.

As used herein, the term "hypertrophic cardiomyopathy" refers to a condition resulting from the right and left heart muscles growing to be different sizes.

As used herein, the term "restrictive cardiomyopathy" refers to a condition characterized by the heart muscle's inability to relax between contractions, which prevents it from filling sufficiently.

The term "ischemic heart disease" refers to any condition in which heart muscle is damaged or works inefficiently because of an absence or relative deficiency of its blood supply; most often caused by atherosclerosis, it includes angina pectoris, acute myocardial infarction, and chronic ischemic heart disease.

"Angina pectoris" refers to chest discomfort caused by inadequate blood flow through the blood vessels (coronary vessels) of the myocardium.

A "myocardial infarction" (heart attack) occurs when an area of heart muscle dies or is damaged because of an inadequate supply of oxygen to that area.

The term "immunosuppressive therapy" is used herein to denote any therapy aimed at decreasing the body's immune response, such as, for example, the production of autoantibodies.

As used herein, the term "immunoadsorption therapy" refers to any treatment that removes antibodies from plasma by binding the target antibodies. Typically, plasma is removed from a subject, contacted with a solid phase-affixed binding partner for the target antibodies under conditions sufficient for binding, followed by return of the plasma to the subject.

A "subject" is a member of any animal species, preferably a mammalian species, optionally a human. The subject can be an apparently healthy individual, an individual suffering from a disease, and an individual being treated for a disease. A "reference subject" or "reference subjects" is/are an individual or a population that serves as a reference against which to assess another individual or population with respect to one or more parameters.

Further with regard to such reference subjects, as described herein, "clinically normal cardiovascular function" means the reference subject has no known or apparent or presently detectable cardiovascular dysfunction and no detectable increase in autoantibodies to endogenous cardiovascular antigens.

A "first degree relative" is either a parent, child, or sibling.

"Samples" that can be assayed using the methods of the present invention include biological fluids, such as whole blood, serum, plasma, synovial fluid, cerebrospinal fluid, bronchial lavage, ascites fluid, bone marrow aspirate, pleural effusion, urine, as well as tumor tissue or any other bodily constituent or any tissue

culture supernatant that could contain the analyte of interest. Samples can be obtained by any appropriate method known in the art.

"Analyte," as used herein, refers to the substance to be detected, which may be suspected of being present in the sample (i.e, the biological sample). The analyte can be any substance for which there exists a naturally occurring specific binding partner or for which a specific binding partner can be prepared. Thus, an analyte is a substance that can bind to one or more specific binding partners in an assay.

A "binding partner," as used herein, is a member of a binding pair, i.e., a pair of molecules wherein one of the molecules binds to the second molecule. Binding partners that bind specifically are termed "specific binding partners." In addition to the antigen and antibody binding partners commonly used in immunoassays, other specific binding partners can include biotin and avidin, carbohydrates and lectins, complementary nucleotide sequences, effector and receptor molecules, cofactors and enzymes, enzyme inhibitors and enzymes, and the like. Furthermore, specific binding partners can include partner(s) that is/are analog(s) of the original specific binding partner, for example, an analyte-analog. Immunoreactive specific binding partners include antigens, antigen fragments, antibodies and antibody fragments, both monoclonal and polyclonal, and complexes thereof, including those formed by recombinant DNA methods.

As used herein, the term "epitope", "epitopes" or "epitopes of interest" refer to a site(s) on any molecule that is recognized and is capable of binding to a complementary site(s) on its specific binding partner. The molecule and specific binding partner are part of a specific binding pair. For example, an epitope can be a polypeptide, protein, hapten, carbohydrate antigen (such as, but not limited to, glycolipids, glycoproteins or lipopolysaccharides) or polysaccharide and its specific binding partner, can be, but is not limited to, an antibody, e.g., an autoantibody. Typically an epitope is contained within a larger antigenic fragment (i.e., region or fragment capable of binding an antibody) and refers to the precise residues known to contact the specific binding partner. It is possible for an antigenic fragment to contain more than one epitope.

As used herein, "specific" or "specificity" in the context of an interaction between members of a specific binding pair (e.g., an antigen and antibody) refers to the

selective reactivity of the interaction. The phrase "specifically binds to" and analogous terms thereof refer to the ability of autoantibodies to specifically bind to (e.g., preferentially react with) an endogeneous cardiovascular antigen and not specifically bind to other entities. Antibodies (including autoantibodies) or antibody fragments that specifically bind to an endogeneous cardiovascular antigen can be identified, for example, by diagnostic immunoassays (e.g., radioimmunoassays ("RIA") and enzyme-linked immunosorbent assays ("ELISAs") (See, for example, Paul, ed., *Fundamental Immunology*, 2nd ed., Raven Press, New York, pages 332-336 (1989)), BIAcore® (biomolecular interaction analysis, e.g., instrument available from BIAcore International AB, a GE Healthcare company, Uppsala, Sweden), KinExA® (Kinetic Exclusion Assay, instrument available from Sapidyne Instruments (Boise, Idaho)) or other techniques known to those of skill in the art. In one embodiment, the term "specifically binds" indicates that the binding preference (e.g., affinity) for the target molecule/sequence is at least about 2-fold, more preferably at least about 5-fold over a non-specific target molecule (e.g., a randomly generated molecule lacking the specifically recognized site(s)).

Autoantibodies which are described as being directed "to different endogenous cardiovascular antigens" means that the autoantibodies have specificity to a different endogenous antigen, and are not merely directed to a different epitope in the same endogenous antigens. However, in addition to the method and panel in the test kit being designed for assessing autoantibodies to different endogenous cardiovascular antigens, optionally, the method and the test kit panel can include means for the detection of one or more autoantibodies which are directed to the same endogenous cardiovascular antigen. In other words, it may be desirable to include in a panel multiple epitopes or antigenic sites from a particular endogenous cardiovascular antigen for detecting autoantibody, particularly when the endogenous cardiovascular antigen is a complex antigenic molecule.

As used herein with reference to autoantibodies to endogenous cardiovascular antigens, or, with reference to endogenous cardiovascular antigens, the term "elevated level" refers to a level in a sample that is higher than a normal level or range, or is higher that an other reference level or range (e.g., earlier or baseline sample). The term "altered level" refers to a level in a sample that is altered (increased or decreased) over

a normal level or range, or over another reference level or range (e.g., earlier or baseline sample). The normal level or range for endogenous cardiovascular antigens and autoantibodies reactive therewith is defined in accordance with standard practice. Because the levels of antibodies in some instances will be very low, a so-called altered level or alteration can be considered to have occurred when there is any net change as compared to the normal level or range, or reference level or range that cannot be explained by experimental error or sample variation. Thus, the level measured in a particular sample will be compared with the level or range of levels determined in similar samples of normal tissue. In this context, "normal tissue" is tissue from an individual with no detectable cardiac pathology, and a "normal" (sometimes termed "control") patient or population is one that exhibits no detectable cardiac pathology. The level of an analyte is said to be "elevated" where the analyte is normally undetectable (e.g., the normal level is zero, or within a range of from about 25 to about 75 percentiles of normal populations), but is detected in a test sample, as well as where the analyte is present in the test sample at a higher than normal level.

A "solid phase," as used herein, refers to any material that is insoluble, or can be made insoluble by a subsequent reaction. The solid phase can be chosen for its intrinsic ability to attract and immobilize a capture agent. Alternatively, the solid phase can have affixed thereto a linking agent that has the ability to attract and immobilize the capture agent. The linking agent can, for example, include a charged substance that is oppositely charged with respect to the capture agent itself or to a charged substance conjugated to the capture agent. In general, the linking agent can be any binding partner (preferably specific) that is immobilized on (attached to) the solid phase and that has the ability to immobilize the capture agent through a binding reaction. The linking agent enables the indirect binding of the capture agent to a solid phase material before the performance of the assay or during the performance of the assay. The solid phase can, for example, be plastic, derivatized plastic, magnetic or non-magnetic metal, glass or silicon, including, for example, a test tube, microtiter well, sheet, bead, microparticle, chip, and other configurations known to those of ordinary skill in the art.

As used herein, term "microparticle" refers to a small particle that is recoverable by ultracentrifugation. Microparticles typically have an average diameter on the order of about 1 micron or less.

The term "capture agent" is used herein to refer to a binding partner that binds to analyte, preferably specifically. Capture agents can be attached to a solid phase. As used herein, the binding of a solid phase-affixed capture agent to analyte forms a "solid phase-affixed complex."

The term "labeled detection agent" is used herein to refer to a binding partner that binds to analyte, preferably specifically, and is labeled with a detectable label or becomes labeled with a detectable label during use in an assay.

A "detectable label" includes a moiety that is detectable or that can be rendered detectable.

As used with reference to a labeled detection agent, a "direct label" is a detectable label that is attached, by any means, to the detection agent.

As used with reference to a labeled detection agent, an "indirect label" is a detectable label that specifically binds the detection agent. Thus, an indirect label includes a moiety that is the specific binding partner of a moiety of the detection agent. Biotin and avidin are examples of such moieties that are employed, for example, by contacting a biotinylated antibody with labeled avidin to produce an indirectly labeled antibody.

As used herein, the term "indicator reagent" refers to any agent that is contacted with a label to produce a detectable signal. Thus, for example, in conventional enzyme labeling, an antibody labeled with an enzyme can be contacted with a substrate (the indicator reagent) to produce a detectable signal, such as a colored reaction product.

As used herein, an "antibody" refers to a protein consisting of one or more polypeptides substantially encoded by immunoglobulin genes or fragments of immunoglobulin genes. This term encompasses polyclonal antibodies, monoclonal antibodies, and fragments thereof, as well as molecules engineered from immunoglobulin gene sequences. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively.

A typical immunoglobulin (antibody) structural unit is known to comprise a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kD) and one "heavy" chain (about 50 - 70 kD). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms "variable light chain (VL)" and "variable heavy chain (VH)" refer to these light and heavy chains respectively.

Antibodies exist as intact immunoglobulins or as a number of well-characterized fragments produced by digestion with various peptidases. Thus, for example, pepsin digests an antibody below the disulfide linkages in the hinge region to produce F(ab')2, a dimer of Fab which itself is a light chain joined to VH-CH1 by a disulfide bond. The F(ab')2 may be reduced under mild conditions to break the disulfide linkage in the hinge region thereby converting the (Fab')2 dimer into a Fab' monomer. The Fab' monomer is essentially a Fab with part of the hinge region (see, Fundamental Immunology, W.E. Paul, ed., Raven Press, N.Y. (1993), for a more detailed description of other antibody fragments). While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that such Fab' fragments may be synthesized de novo either chemically or by utilizing recombinant DNA methodology.

Thus, the term "antibody," as used herein also includes antibody fragments either produced by the modification of whole antibodies or synthesized de novo using recombinant DNA methodologies. Antibodies include single chain antibodies (antibodies that exist as a single polypeptide chain), single chain Fv antibodies (sFv or scFv), in which a variable heavy and a variable light chain are joined together (directly or through a peptide linker) to form a continuous polypeptide. The single chain Fv antibody is a covalently linked VH-VL heterodimer which may be expressed from a nucleic acid including VH- and VL- encoding sequences either joined directly or joined by a peptide-encoding linker (Huston, et al., (1988) Proc. Nat. Acad. Sci. USA, 85: 5879-5883). While the VH and VL are connected to each as a single polypeptide chain, the VH and VL domains associate non-covalently. The scFv antibodies and a number of other structures convert the naturally aggregated, but chemically separated, light and heavy polypeptide chains from an antibody V region into a molecule that

folds into a three dimensional structure substantially similar to the structure of an antigen-binding site are known to those of skill in the art (see e.g., U.S. Patent Nos. 5,091,513, 5,132,405, and 4,956,778).

An "autoantibody" is an antibody that binds to an analyte that is naturally occurring in the individual in which the antibody is produced. An autoantibody to an endogenous cardiovascular antigen is an autoantibody that binds an endogenous cardiovascular antigen.

As used herein, the singular forms "a", "an" and "the" include plural references unless the context clearly dictates otherwise.

As used herein, the term "about" refers to approximately a +/-10% variation from the stated value. It is to be understood that such a variation is always included in any given value provided herein, whether or not it is specifically referred to.

Sample Collection and Processing

The assay methods of the invention are generally carried out on samples derived from an animal, preferably a mammal, and more preferably a human.

The methods of the invention can be carried out using any sample that may contain autoantibodies to different endogenous cardiovascular antigens. Convenient samples include, for example, blood, serum, and plasma.

The sample may be pretreated, as necessary or desired, by dilution in an appropriate buffer solution or other solution, or optionally may be concentrated. Any of a number of standard aqueous buffer solutions, employing any of a variety of buffers, such as phosphate, Tris, or the like, optionally at physiological pH, can be used.

Assay of Autoantibodies to Different Endogenous Cardiovascular Antigens

Among other things the description herein provides an assay for autoantibodies to endogenous cardiovascular antigens. In one embodiment, this method provides for use of the assay in assessing the risk of whether a subject has or might develop a cardiovascular autoimmune disease and optionally comprises:

(a) obtaining a sample from the subject;

(b) measuring the levels of autoantibodies to each of a plurality of different endogenous cardiovascular antigens in the subject's sample;

(c) comparing these levels of autoantibodies with the level of the same autoantibodies measured in samples from reference subjects with clinically normal cardiovascular function, or from reference subjects having cardiovascular disease, and

(d) identifying the risk that the subject has or might develop a cardiovascular autoimmune disease based on the comparison in step (c).

In this method, risk is considered to be present when levels of autoantibodies to at least two of the different endogenous cardiovascular antigens:

(i) are elevated in the subject's sample as compared to in samples from reference subjects with clinically normal cardiovascular function, and/or

(ii) are elevated in or at about the same level in the subject's sample as compared to in samples from reference subjects having cardiovascular disease.

Cardiovascular Autoimmune Disease: Myocarditis, Cardiomyopathy, and Ischemic Heart Disease

In particular embodiments, the invention provides a method of screening for a subject having, or at risk of having, myocarditis, ischemic heart disease, or cardiomyopathy. In variations of these embodiments the cardiomyopathy is not dilated cardiomyopathy. Thus, for example, the method can be employed to screen for subjects having, or at risk of having, hypertrophic cardiomyopathy and/or restrictive cardiomyopathy.

The method entails assaying a sample from the subject for autoantibodies to each of a plurality of different endogenous cardiovascular antigens, wherein the presence of an elevated level of autoantibodies to at least two of the different endogenous cardiovascular antigens indicates the presence of, or risk of, cardiovascular autoimmune disease. This method can be performed in conjunction with one or more other tests, including but not limited to physical examination, and/or the taking of a medical history to allow a differential diagnosis of, e.g., myocarditis, ischemic heart disease, or hypertrophic or restrictive cardiomyopathy. The various tests and parameters employed in diagnosing these disorders are well known to those of skill in the art.

These methods can be carried out on samples from asymptomatic subjects or subjects having one or more risk factors associated with, or symptoms of, cardiovascular autoimmune disease. For example, the subject may have an autoimmune disease, high blood pressure, or may have close (e.g., first-degree) relative with a heritable cardiovascular autoimmune disease, such as hypertophic cardiomyopathy, or with an autoimmune disease that may deleteriously impact cardiovascular function (e.g., diabetes, rheumatic heart disease, or lupus).

Autoantibodies to endogenous cardiovascular antigens can be detected and quantified by any convenient means. Examples of various immunoassay formats suitable for this purpose are described below. The assays are scored in accordance with standard practice.

In particular embodiments, when a subject is determined to have an elevated level of autoantibodies to at least two different endogenous cardiovascular antigens, the subject optionally is assessed for one or more additional indicators of cardiovascular autoimmune disease such as myoglobin, CK-MB, BNP, CRP, Troponin-I, Troponin-T, blood oxygen level, cardiac imaging, electrocardiography and the like.

Assay of Autoantibodies to Different Endogenous Cardiovascular Antigens in Conjunction With Assay of Endogenous Cardiovascular Antigens

Among other things, an assay for autoantibodies to the endogenous cardiovascular antigen troponin is described in US 11/588073 entitled "Assay For Cardiac Troponin Autoantibodies" filed October 26, 2006. As described therein, one method of assessing risk of cardiac pathology entails (a) assaying a sample for cardiac troponin, and (b) assaying a sample for an autoantibody specific for cardiac troponin, where the presence of an elevated level of cardiac troponin and/or an elevated level of cardiac troponin-reactive autoantibody indicates an elevated risk of cardiac pathology.

In the context of the present invention, the presence of an elevated level of cardiac troponin and an elevated level of autoantibodies to at least two of the different endogenous cardiovascular antigens can be employed as an indicator of elevated risk of cardiovascular autoimmune disease. In one embodiment, the endogenous cardiovascular antigens being assayed along with the autoantibodies may include cardiac troponin. In other embodiments, the endogenous cardiovascular antigens being assayed may include antigens other than cardiac troponin (e.g., as where this is not

tested for as part of the panel assay). Optionally the specific endogenous cardiovascular antigens being assessed in an assay may be the same as, or different than, the plurality of endogenous cardiovascular antigens against the autoantibodies identified by the assay are directed.

Moreover, with endogenous cardiovascular antigens other than cardiac troponin, for some antigens and/or at some stages of cardiovascular autoimmune disease, the level of the endogenous cardiovascular antigen will decrease rather than increase over time and/or compared to another reference (e.g., normal, or disease cut-off) level. Such a change provides valuable diagnostic or prognostic information. Thus, one embodiment of the invention is directed to a method of assessing risk of cardiovascular autoimmune disease wherein the method entails: (a) assaying a sample for the levels of one or more endogenous cardiovascular antigens, and (b) assaying a sample for the levels of autoantibodies to each of a plurality of different endogenous cardiovascular antigens, where the presence of an altered level of endogenous cardiovascular antigen and/or elevated levels of autoantibodies to at least two of said different endogenous cardiovascular antigens indicates an elevated risk of cardiovascular autoimmune disease.

In another embodiment, a method for assessing the risk of whether a subject has or might develop a cardiovascular autoimmune disease comprises:

(a) obtaining a sample from the subject;

(b) measuring the levels of one or more endogenous cardiovascular antigens in the subject's sample;

(c) measuring the levels of autoantibodies to each of a plurality of different endogenous cardiovascular antigens in the subject's sample;

(d) comparing the levels of the one or more endogenous cardiovascular antigens with the level of the same endogenous cardiovascular antigen measured in samples from reference subjects with clinically normal cardiovascular function, in samples from reference subjects having cardiovascular disease, or in the subject's sample obtained at an earlier time;

(e) comparing the levels of autoantibodies with the level of the same autoantibody measured in samples from reference subjects with clinically normal

cardiovascular function, in samples from reference subjects having cardiovascular disease, or in the subject's sample obtained at an earlier time; and

(f) identifying the risk that the subject has or might develop a cardiovascular autoimmune disease based on the comparison in steps (d) and (e).

According to such a method, the risk is present when:

(i) in the comparison in step (d) the levels of the one or more endogenous cardiovascular antigens are altered in the subject's sample as compared to in samples from reference subjects with clinically normal cardiovascular function, and/or are altered in or at about the same level in the subject's sample as compared to in the subject's sample obtained at an earlier time; and/or

(ii) in the comparison in step (e) the levels of the autoantibodies to at least two of the different endogenous cardiovascular antigens are elevated in the subject's sample as compared to in samples from reference subjects with clinically normal cardiovascular function, and/or are elevated in or at about the same in the subject's sample as compared to in samples from reference subjects having cardiovascular disease.

These methods can be carried out on samples from asymptomatic subjects or subjects with one or more symptoms of cardiovascular autoimmune disease. For example, the subject may have chest pain or some other indication of myocardial infarction. The method optionally can be carried out where the determinations of steps (b) and (c) are done simultaneously, or are done sequentially (optionally in any order). Also, the assay for autoantibodies to endogenous cardiovascular antigens can be carried out in the absence of any assay for endogenous cardiovascular antigens. The assay for autoantibodies to endogenous cardiovascular antigens can be carried out using the same sample or a different sample from the same subject as that employed for assay of endogenous cardiovascular antigens. If a different sample is used, it will generally be of the same type (e.g., blood) and taken at approximately the same time as the sample which was assayed for the endogenous cardiovascular antigens.

Autoantibodies to endogenous cardiovascular antigens can be detected and quantified by any of a number of methods well known to those of skill in the art. These include any of a number of immunological methods such as fluid or gel precipitin reactions, immunodiffusion (single or double), affinity chromatography,

immunoelectrophoresis, radioimmunoassay (RIA), enzyme-linked immunosorbent assays (ELISAs), immunofluorescent assays, Western blotting, and the like. Immunoassays useful in the methods of the invention are discussed in greater detail below.

The assays are scored in accordance with standard practice and optionally include the use of positive and/or negative controls and/or or standards containing known concentrations of antibodies to endogenous cardiovascular antigens. The level of autoantibodies to endogenous cardiovascular antigens optionally is compared with a control level or control range, which can be determined when the assay is carried out or, more conveniently, can be predetermined. Any increase in the test sample relative to the control level or range in terms of autoantibody levels, or any alteration (increase or decrease) in terms of endogenous cardiovascular antigen levels, can be assessed for significance by conventional statistical methods. The presence of an elevated level of autoantibodies to endogenous cardiovascular antigens indicates that such autoantibodies may negatively interfere with measurement of the endogenous cardiovascular antigens, rendering such value unreliable with respect to assessing the risk of cardiac pathologies, such as myocardial infarction.

In particular embodiments, when a subject is determined to have an elevated level of autoantibodies to endogenous cardiovascular antigens, optionally the subject is assessed for one or more additional indicators of cardiac pathology such as myoglobin, CK-MB (creatine kinase muscle-brain), BNP (brain natriuretic peptide), CRP (C reactive protein), cardiac troponin I (cTnI), cardiac troponin T (cTnT), blood oxygen level, cardiac imaging, electrocardiography and the like. However, such testing can optionally be carried out even when there has been no prior detection of an elevated level of autoantibodies. For example, even in the absence of detection of elevated autoantibodies, the methods of the present invention can also be accompanied by measurement of one or more markers associated with heart disease. Such markers include but are not limited to pregnancy-associated plasma protein A (PAPP-A), IL-8, IL-10, interleukin-18 (IL-18/IL-18b), ischemic modified albumin (IMA), ICAM-1 (intercellular cell adhesion molecule-1), VCAM-1 (vascular cell adhesion molecule-1), fatty acid binding protein (FABP), E-selectin, P-selectin, fibrinogen, serum amyloid A (SAA), MPO (myeloperoxidase), LpPLA2 (lipoprotein-associated phospholipase A2),

GP-BB (glycogen phosphorylase isoenzyme BB), IL1RA, TAFI (thrombin activable fibrinolysis inhibitor), soluble fibrin, anti-oxLDL (antibodies against oxidized low density lipoprotein), MCP-1 (monocyte chemoattractant protein-1), procoagulant tissue factor (TF), MMP-9 (matrix metalloproteinase 9), Ang-2 (angiopoietin-2), bFGF (basic fibroblast growth factor), VLDL (very low density lipoprotein), PAI-1 (plasminogen activator inhibitor-1).

A subject determined to have an elevated level of autoantibodies to at least two different endogenous cardiovascular antigens may also be treated, e.g., for myocardial infarction in accordance with standard practice.

Relatives of Individuals with Autoimmune Disease

The methods of the invention can be carried out to identify cardiovascular autoimmune disease or risk thereof in subjects who have an autoimmune disease or who are related to an individual with an autoimmune disease. Subjects who are, e.g., first-degree or second-degree relatives of an individual with an autoimmune disease can be assessed using the methods of the invention.

The method entails assaying a sample from the subject for an autoantibodies to a plurality of different endogenous cardiovascular antigens, wherein the presence of an elevated level of autoantibodies to at least two different endogenous cardiovascular antigens indicates the presence of, or risk of, cardiovascular autoimmune disease. This method can be performed in conjunction with one or more other tests, physical examination, and/or the taking of a medical history to allow a differential diagnosis of, e.g., myocarditis, ischemic heart disease, or dilated, hypertrophic, or restrictive cardiomyopathy. The various tests and parameters employed in diagnosing these disorders are well known to those of skill in the art.

These methods can be carried out on samples from asymptomatic subjects or subjects having one or more risk factors associated with, or symptoms of, cardiovascular autoimmune disease.

Autoantibodies to different endogenous cardiovascular antigens can be detected and quantified by any convenient means. Examples of various immunoassay formats suitable for this purpose are described below. The assays are scored in accordance with standard practice.

In particular embodiments, when a subject is determined to have an elevated level of autoantibodies to at least two different endogenous cardiovascular antigens, the subject optionally is assessed for one or more additional indicators of cardiovascular autoimmune disease such as myoglobin, CK-MB, BNP, CRP, Troponin-I, Troponin-T, blood oxygen level, cardiac imaging, electrocardiography and the like.

Method of Identifying Candidates for Immunosuppressive or Immunoabsorption Therapies

In particular embodiments, the invention also provides a method of determining whether a subject having, or at risk for, a cardiovascular autoimmune disease is a candidate for immunosuppressive therapy or immunoabsorption therapy. Generally, the subject is one who has experienced some symptom of cardiovascular autoimmune disease or who has actually been diagnosed as having, or being at risk for, a cardiovascular autoimmune disease.

The method entails assaying a sample from the subject for autoantibodies to a plurality of endogenous cardiovascular antigens, wherein the presence of elevated levels of autoantibodies to at least two of the different endogenous cardiovascular antigens indicates that autoimmunity may be contributing to the subject's cardiovascular autoimmune disease or risk thereof. This method can be performed in conjunction with one or more other tests, physical examination, and/or the taking of a medical history in accordance with standard practice for diagnosing cardiac pathologies and/or autoimmune diseases.

In one embodiment, this method optionally comprises identifying a patient with cardiovascular disease as eligible to receive autoimmune therapy by the steps of:

(a) obtaining a sample from the cardiovascular disease patient;

(b) measuring the levels of autoantibodies to each of a plurality of different endogenous cardiovascular antigens in the subject's sample;

(c) comparing the levels with the level of the same autoantibodies measured in samples from reference subjects with clinically normal cardiovascular function, or from reference subjects having autoimmune disease; and

(d) identifying the cardiovascular disease patient as eligible to receive autoimmune therapy based on the comparison in step (c).

In such a method, the patient is eligible to receive autoimmune therapy when levels of autoantibodies to at least two of the different endogenous cardiovascular antigens:

(i) are elevated in the cardiovascular disease patient's sample as compared to in samples from reference subjects with clinically normal cardiovascular function, and/or

(ii) are elevated in or at about the same in the cardiovascular disease patient's sample as compared to in samples from reference subjects having autoimmune disease.

Autoantibodies to endogenous cardiovascular antigens can be detected and quantified by any convenient means, including any of those described herein. The assays are scored in accordance with standard practice.

A subject determined to have an elevated level of autoantibodies to at least two different endogenous cardiovascular antigens may also be treated with immunosuppressive therapy or immunoabsorption therapy in accordance with standard practice.

Immunoassay Methods - In General

The immunoassay methods of the invention can be carried out in any of a wide variety of formats. For a general review of immunoassays, see Methods in Cell Biology Volume 37: Antibodies in Cell Biology, Asai, ed. Academic Press, Inc. New York (1993); Basic and Clinical Immunology 7th Edition, Stites & Terr, eds. (1991), which is incorporated by reference in its entirety.

In particular embodiments, an immunoassay method of the invention can be performed by contacting a sample with an endogenous cardiovascular antigen, under conditions sufficient for binding of the endogenous cardiovascular antigen to any reactive autoantibody present in the sample. Autoantibodies are detected/quantitated by detecting complex(es) comprising the endogenous cardiovascular antigen bound to the reactive autoantibody. Such assays can be homogeneous or heterogeneous (i.e., employing a solid phase). In heterogeneous assays, a capture agent that binds to the analyte (here, autoantibodies to endogenous cardiovascular antigens) is typically affixed to a solid phase.

Autoantibodies to endogenous cardiovascular antigens can be measured in a non-competitive immunoassay, wherein the amount of endogenous cardiovascular

antigen bound to autoantibody to endogenous cardiovascular antigen is positively correlated with the concentration of autoantibody to endogenous cardiovascular antigen present in the sample.

Thus, for example, the method can be carried out as an agglutination assay in which the sample is contacted with an endogenous cardiovascular antigen affixed to a solid phase, such as a microparticle. The binding of an autoantibody to endogenous cardiovascular antigen that is present in the sample to the microparticles results in the agglutination of those microparticles, which can be detected, for example, by visual inspection of the sample. The microparticles can be colored or labeled, if desired, to facilitate detection of agglutination. The degree of agglutination is positively correlated with the concentration of autoantibody to endogenous cardiovascular antigen present in the sample.

In other embodiments, the sample is contacted with the endogenous cardiovascular antigens (which may, but need not, be affixed to a solid phase) and also contacted with a species-specific antibody, wherein the species-specific antibody is specific for the species from which the sample was obtained. Means for correcting interference generated by such autoantibodies is independently described in the U.S. Patent Application Serial Number 60/854569, which is incorporated herein by reference in its entirety for its teachings regarding same. This step is carried out under conditions sufficient for specific binding of the species-specific antibody to any autoantibody to endogenous cardiovascular antigen that is present. Autoantibodies are detected/quantitated by detecting complex(es) comprising the endogenous cardiovascular antigen bound to the reactive autoantibody, which is bound to species-specific antibody. The sample may be contacted with the endogenous cardiovascular antigen and the species-specific antibody simultaneously or sequentially, in any order. Regardless of the order of contact, if autoantibodies to endogenous cardiovascular antigens are present in the sample, a complex forms that contains the antibodies "sandwiched" between the endogenous cardiovascular antigen and the species-specific antibody.

For example, in one format of a sandwich immunoassay, an embodiment of the invention, the endogenous cardiovascular antigen is affixed to a solid phase, binding of the endogenous cardiovascular antigen to any autoantibody to endogenous

cardiovascular antigen present in the sample forms a solid phase-affixed complex, and detecting comprises detecting a signal from the solid phase-affixed complex. In particular embodiments of this format, the solid phase-affixed complex is detected using a species-specific antibody that is directly or indirectly labeled. The bound entities are separated, if necessary, from free labeled species-specific antibody, typically by washing, and the signal from the bound label is detected.

In another format of a sandwich immunoassay, an embodiment of the invention, the species-specific antibody is affixed to a solid phase, binding of the species-specific antibody to any autoantibody to endogenous cardiovascular antigen present in the sample forms a solid phase-affixed complex, which is then detected. In certain embodiments, the solid phase-affixed complex is detected using an endogenous cardiovascular antigen that is directly or indirectly labeled. The bound entities are separated, if necessary, from free labeled endogenous cardiovascular antigen, typically by washing, and the signal from the bound label is detected.

Autoantibodies to endogenous cardiovascular antigens can also be measured in competitive immunoassay, wherein the signal is negatively correlated with the concentration of autoantibody to endogenous cardiovascular antigen present in the sample. In an example of a competitive format, the sample is contacted with an endogenous cardiovascular antigen (which may, but need not, be affixed to a solid phase) and also contacted with a labeled (directly or indirectly) antibody to endogenous cardiovascular antigen. This step is carried out under conditions sufficient for specific binding of the labeled antibody to endogenous cardiovascular antigen with the endogenous cardiovascular antigen. Autoantibodies in the sample that are specific for endogenous cardiovascular antigen can compete with the labeled antibody to endogenous cardiovascular antigen for binding to the endogenous cardiovascular antigen. Accordingly, the higher the level of autoantibody to endogenous cardiovascular antigen in a sample, the lower is the binding of labeled antibody to the endogenous cardiovascular antigen with the endogenous cardiovascular antigen.

The sample may be contacted with the endogenous cardiovascular antigen and the labeled antibody to endogenous cardiovascular antigen simultaneously or sequentially, in any order.

Competitive immunoassays of this type can be conveniently carried out using a solid phase-affixed endogenous cardiovascular antigen. In this case, binding of the endogenous cardiovascular antigen to labeled antibody to endogenous cardiovascular antigen, or to any autoantibody to endogenous cardiovascular antigen that is present in the sample forms a solid phase-affixed complex, and detection entails detecting a signal from the solid phase-affixed complex. The bound entities are separated, if necessary, from free labeled antibody to endogenous cardiovascular antigen, typically by washing, and the signal from the bound label is detected.

Exemplary immunoassays for autoantibodies are described, for example, U.S. Patent Application Serial Numbers 11/588,073, 11/934,688 and 61/015,449, which are incorporated herein by reference in their entireties for their teachings regarding same.

Capture Agent

Capture agents useful in the immunoassay methods of the invention include those that bind to endogenous cardiovascular antigen, or autoantibody to endogenous cardiovascular antigen, and can be affixed to a solid phase. Convenient capture agents include endogenous cardiovascular antigens and species-specific antibodies, wherein the species-specific antibody is specific for the species from which the sample was obtained. As those of skill in the art appreciate, each endogenous cardiovascular antigen represents a specific capture agent because it binds (captures) the corresponding autoantibody to endogenous cardiovascular antigen. By contrast, species-specific antibodies represent a non-specific capture agent because such antibodies bind autoantibodies, regardless of specificity. In a sandwich immunoassay, a non-specific capture agent is typically employed with a labeled detection agent that specifically binds the analyte. Thus, for example, solid phase affixed species-specific antibodies can be used in conjunction with a labeled endogenous cardiovascular antigen to specifically detect autoantibodies to endogenous cardiovascular antigens.

Endogenous Cardiovascular Antigens

Endogenous cardiovascular antigens useful in the immunoassay methods and kits of the invention (e.g., for binding, or as calibrators or controls) include but are not limited to the following endogenous antigens: α1-adrenoceptor; angiotensin-1 receptor;

annexin V; brain natriuretic peptide (BNP); cardiac troponins; cardiolipin; myosin; tropomyosin; cytoplasmic neutrophils; endothelial receptor of protein C; Factor VIII; grehlin; halogenated protein; nitrated protein; heat shock proteins (HSPs); myeloperoxidase (MPO); oxidized low-density lipoprotein (LDL); placental growth factor (P*l*GF); phospholipids; proteinase-3; prothrombin; Purkinje fibers; sarcolemmal Na-K-ATPase; β1-adrenoceptor; β2-adrenoceptor; β-2-glycoprotein-1; and tissue-type plasminogen activator.

In particular embodiments, the endogenous cardiovascular antigen is a endogenous cardiovascular antigen amino acid sequence that can be derived from any endogenous cardiovascular antigen-like polypeptide from any organism. Endogenous cardiovascular antigen amino acid sequences useful in the invention are generally those derived from vertebrates, preferably from birds or mammals, more preferably from animals having research or commercial value or value as pets, such as mice, rats, guinea pigs, rabbits, cats, dogs, chickens, pigs, sheep, goats, cows, horses, as well as monkeys and other primates. In particular embodiments, the endogenous cardiovascular antigen amino acid sequence is derived from a human polypeptide.

The methods of the invention can employ full-length endogenous cardiovascular antigens or one or more fragments thereof. Fragments will generally have at least one epitope to which an autoantibody can bind. Such fragments can have a length, e.g., of about 125, 100, 75, 50, 25, or 15 amino acids or a length that falls within a range with endpoints defined by any of these values (e.g., 15-125, 25-100, 50-75, 15-100, etc.). Those of skill in the art readily appreciate that the use of an endogenous cardiovascular antigen having a larger number of natural epitopes (e.g., a full-length endogenous cardiovascular antigen) will generally provide a more comprehensive measurement of autoantibodies of different specificities than the use of an endogenous cardiovascular antigen having a smaller number of natural epitopes. Accordingly, it is generally preferable to employ an endogenous cardiovascular antigen that has a substantially native conformation or one or more peptides comprising endogenous cardiovascular antigen epitopes reactive with the autoantibody.

The endogenous cardiovascular antigen amino acid sequence can be a wild-type amino acid sequence or an amino acid sequence variant of the corresponding region of a wild-type polypeptide. In certain embodiments, endogenous cardiovascular antigens

include a wild-type endogenous cardiovascular antigen amino acid sequence or an endogenous cardiovascular antigen amino acid sequence containing conservative amino acid substitutions, as defined above.

In addition to the amino acid sequences described above, endogenous cardiovascular antigens useful in the invention can include other amino acid sequences, including those from heterologous proteins. Accordingly, the invention encompasses fusion polypeptides in which an endogenous cardiovascular antigen amino acid sequence is fused, at either or both ends, to amino acid sequence(s) from one or more heterologous proteins. Examples of additional amino acid sequences often incorporated into proteins of interest include a signal sequence, which facilitates purification of the protein, and an epitope tag, which can be used for immunological detection or affinity purification.

Endogenous cardiovascular antigen polypeptides according to the invention can be synthesized using methods known in the art, such as for example exclusive solid phase synthesis, partial solid phase synthesis, fragment condensation, and classical solution synthesis. See, e.g., Merrifield, J. Am. Chem. Soc., 85:2149 (1963). For a description of solid phase peptide synthesis procedures, see John Morrow Stewart and Janis Dillaha Young, Solid Phase Peptide Syntheses (2nd Ed., Pierce Chemical Company, 1984).

Endogenous cardiovascular antigen polypeptides can also produced using recombinant techniques. In certain embodiments, the sequence of an endogenous cardiovascular antigen coding region is used as a guide to design a synthetic nucleic acid molecule encoding the endogenous cardiovascular antigen polypeptide that can be incorporated an expression vector. Methods for constructing synthetic genes are well-known to those of skill in the art. See, e.g., Dennis, M. S., Carter, P. and Lazarus, R. A., Proteins: Struct. Funct. Genet., 15:312–321 (1993).

The expression vector includes one or more control sequences capable of effecting and/or enhancing the expression of an operably linked polypeptide coding sequence. Control sequences that are suitable for expression in prokaryotes, for example, include a promoter sequence, an operator sequence, and a ribosome binding site. Control sequences for expression in eukaryotic cells include a promoter, an enhancer, and a transcription termination sequence (i.e., a polyadenylation signal).

An expression vector according to the invention can also include other sequences, such as, for example, nucleic acid sequences encoding a signal sequence or an amplifiable gene. A signal sequence can direct the secretion of a polypeptide fused thereto from a cell expressing the protein. In the expression vector, nucleic acid encoding a signal sequence is linked to a polypeptide coding sequence so as to preserve the reading frame of the polypeptide coding sequence. The inclusion in a vector of a gene complementing an auxotrophic deficiency in the chosen host cell allows for the selection of host cells transformed with the vector.

A wide variety of host cells are available for propagation and/or expression of vectors. Examples include prokaryotic cells (such as *E. coli* and strains of Bacillus, Pseudomonas, and other bacteria), yeast or other fungal cells (including *S. cerevesiae* and *P. pastoris*), insect cells, plant cells, and phage, as well as higher eukaryotic cells (such as human embryonic kidney cells and other mammalian cells).

Vectors expressing endogenous cardiovascular antigen can be introduced into a host cell by any convenient method, which will vary depending on the vector-host system employed. Generally, a vector is introduced into a host cell by transformation or infection (also known as "transfection") with a virus (e.g., phage) bearing the vector. If the host cell is a prokaryotic cell (or other cell having a cell wall), convenient transformation methods include the calcium treatment method described by Cohen, et al., (1972) Proc. Natl. Acad. Sci., USA, 69:2110-14. If a prokaryotic cell is used as the host and the vector is a phagemid vector, the vector can be introduced into the host cell by transfection. Yeast cells can be transformed using polyethylene glycol, for example, as taught by Hinnen (1978) Proc. Natl. Acad. Sci, USA, 75:1929-33. Mammalian cells are conveniently transformed using the calcium phosphate precipitation method described by Graham, et al. (1978) Virology, 52:546 and by Gorman, et al. (1990) DNA and Prot. Eng. Tech., 2:3-10. However, other known methods for introducing DNA into host cells, such as nuclear injection, electroporation, protoplast fusion, and other means also are acceptable for use in the invention.

Expression of endogenous cardiovascular antigen from a transformed host cell entails culturing the host cell under conditions suitable for cell growth and expression and recovering the expressed polypeptides from a cell lysate or, if the polypeptides are secreted, from the culture medium. In particular, the culture medium contains

appropriate nutrients and growth factors for the host cell employed. The nutrients and growth factors are, in many cases, well known or can be readily determined empirically by those skilled in the art. Suitable culture conditions for mammalian host cells, for instance, are described in Mammalian Cell Culture (Mather ed., Plenum Press 1984) and in Barnes and Sato (1980) Cell 22:649.

In addition, the culture conditions should allow transcription, translation, and protein transport between cellular compartments. Factors that affect these processes are well-known and include, for example, DNA/RNA copy number; factors that stabilize DNA; nutrients, supplements, and transcriptional inducers or repressors present in the culture medium; temperature, pH and osmolality of the culture; and cell density. The adjustment of these factors to promote expression in a particular vector-host cell system is within the level of skill in the art. Principles and practical techniques for maximizing the productivity of in vitro mammalian cell cultures, for example, can be found in Mammalian Cell Biotechnology: a Practical Approach (Butler ed., IRL Press (1991).

Any of a number of well-known techniques for large- or small-scale production of proteins can be employed in expressing the polypeptides of the invention. These include, but are not limited to, the use of a shaken flask, a fluidized bed bioreactor, a roller bottle culture system, and a stirred tank bioreactor system. Cell culture can be carried out in a batch, fed-batch, or continuous mode.

Methods for recovery of recombinant proteins produced as described above are well-known and vary depending on the expression system employed. A polypeptide including a signal sequence can be recovered from the culture medium or the periplasm. Polypeptides can also be expressed intracellularly and recovered from cell lysates.

The expressed polypeptides can be purified from culture medium or a cell lysate by any method capable of separating the polypeptide from one or more components of the host cell or culture medium. Typically, the polypeptide is separated from host cell and/or culture medium components that would interfere with the intended use of the polypeptide. As a first step, the culture medium or cell lysate is usually centrifuged or filtered to remove cellular debris. The supernatant is then typically concentrated or diluted to a desired volume or diafiltered into a suitable buffer to condition the preparation for further purification.

The polypeptide can then be further purified using well-known techniques. The technique chosen will vary depending on the properties of the expressed polypeptide. If, for example, the polypeptide is expressed as a fusion protein containing an epitope tag or other affinity domain, purification typically includes the use of an affinity column containing the cognate binding partner. For instance, polypeptides fused with green fluorescent protein, hemagglutinin, or FLAG epitope tags or with hexahistidine or similar metal affinity tags can be purified by fractionation on an affinity column.

Antibodies

Antibodies useful in the immunoassay methods and kits of the invention include polyclonal and monoclonal antibodies directed against either the endogenous cardiovascular antigen, or the autoantibody to the endogenous cardiovascular antigen. Such polyclonal and monoclonal antibodies can be prepared by any means known in the art. Antibodies to endogenous cardiovascular antigens are known in the art and/or are commercially available, and any of these can be used. Polyclonal antibodies are raised by injecting (e.g., subcutaneous or intramuscular injection) an immunogen into a suitable non-human mammal (e.g., a mouse or a rabbit). Generally, the immunogen should induce production of high titers of antibody with relatively high affinity for the target antigen.

If desired, the endogenous cardiovascular antigen may be conjugated to a carrier protein by conjugation techniques that are well known in the art. Commonly used carriers include keyhole limpet hemocyanin (KLH), thyroglobulin, bovine serum albumin (BSA), and tetanus toxoid. The conjugate is then used to immunize the animal.

The antibodies are then obtained from blood samples taken from the animal. The techniques used to produce polyclonal antibodies are extensively described in the literature (see, e.g., Methods of Enzymology, "Production of Antisera With Small Doses of Immunogen: Multiple Intradermal Injections," Langone, et al. eds. (Acad. Press, 1981)). Polyclonal antibodies produced by the animals can be further purified, for example, by binding to and elution from a matrix to which the target antigen is bound. Those of skill in the art will know of various techniques common in the immunology arts for purification and/or concentration of polyclonal, as well as

monoclonal, antibodies see, for example, Coligan, et al. (1991) Unit 9, Current Protocols in Immunology, Wiley Interscience.

For many applications, monoclonal antibodies (mAbs) are preferred. The general method used for production of hybridomas secreting mAbs is well known (Kohler and Milstein (1975) Nature, 256:495). Briefly, as described by Kohler and Milstein, the technique entailed isolating lymphocytes from regional draining lymph nodes of five separate cancer patients with either melanoma, teratocarcinoma or cancer of the cervix, glioma or lung, (where samples were obtained from surgical specimens), pooling the cells, and fusing the cells with SHFP-1. Hybridomas were screened for production of antibody that bound to cancer cell lines. Confirmation of specificity among mAbs can be accomplished using routine screening techniques (such as the enzyme-linked immunosorbent assay, or "ELISA") to determine the elementary reaction pattern of the mAb of interest.

As used herein, the term "antibody" encompasses antigen-binding antibody fragments, e.g., single chain antibodies (scFv or others), which can be produced/selected using phage display technology or yeast display technology. The ability to express antibody fragments on the surface of viruses that infect bacteria (bacteriophage or phage) makes it possible to isolate a single binding antibody fragment, e.g., from a library of greater than 1010 nonbinding clones. To express antibody fragments on the surface of phage (phage display), an antibody fragment gene is inserted into the gene encoding a phage surface protein (e.g., pIII) and the antibody fragment-pIII fusion protein is displayed on the phage surface (McCafferty et al. (1990) Nature, 348: 552-554; Hoogenboom et al. (1991) Nucleic Acids Res. 19: 4133-4137).

Since the antibody fragments on the surface of the phage are functional, phage-bearing antigen-binding antibody fragments can be separated from non-binding phage by antigen affinity chromatography (McCafferty et al. (1990) Nature, 348: 552-554). Depending on the affinity of the antibody fragment, enrichment factors of 20-fold - 1,000,000-fold are obtained for a single round of affinity selection. By infecting bacteria with the eluted phage, however, more phage can be grown and subjected to another round of selection. In this way, an enrichment of 1000-fold in one round can become 1,000,000-fold in two rounds of selection (McCafferty et al. (1990) Nature, 348: 552-554). Thus, even when enrichments are low (Marks et al. (1991) J. Mol. Biol.

222: 581-597), multiple rounds of affinity selection can lead to the isolation of rare phage. Since selection of the phage antibody library on antigen results in enrichment, the majority of clones bind antigen after as few as three to four rounds of selection. Thus only a relatively small number of clones (several hundred) need to be analyzed for binding to antigen.

Human antibodies can be produced without prior immunization by displaying very large and diverse V-gene repertoires on phage (Marks et al. (1991) J. Mol. Biol. 222: 581-597). In one embodiment, natural VH and VL repertoires present in human peripheral blood lymphocytes are isolated from unimmunized donors by PCR. The V-gene repertoires can be spliced together at random using PCR to create a scFv gene repertoire which can be cloned into a phage vector to create a library of 30 million phage antibodies (Id.). From a single "naïve" phage antibody library, binding antibody fragments have been isolated against more than 17 different antigens, including haptens, polysaccharides, and proteins (Marks et al. (1991) J. Mol. Biol. 222: 581-597; Marks et al. (1993). Bio/Technology. 10: 779-783; Griffiths et al. (1993) EMBO J. 12: 725-734; Clackson et al. (1991) Nature. 352: 624-628). Antibodies have been produced against self proteins, including human thyroglobulin, immunoglobulin, tumor necrosis factor, and CEA (Griffiths et al. (1993) EMBO J. 12: 725-734). The antibody fragments are highly specific for the antigen used for selection and have affinities in the 1 nM to 100 nM range (Marks et al. (1991) J. Mol. Biol. 222: 581-597; Griffiths et al. (1993) EMBO J. 12: 725-734). Larger phage antibody libraries result in the isolation of more antibodies of higher binding affinity to a greater proportion of antigens.

As those of skill in the art readily appreciate, antibodies can be prepared by any of a number of commercial services (e.g., Berkeley Antibody Laboratories, Bethyl Laboratories, Anawa, Eurogenetec, etc.).

Solid Phase

For embodiments of the invention that employ a solid phase as a support for the capture agent, the solid phase can be any suitable material with sufficient surface affinity to bind a capture agent. Useful solid supports include: natural polymeric carbohydrates and their synthetically modified, crosslinked, or substituted derivatives, such as agar, agarose, cross-linked alginic acid, substituted and cross-linked guar gums,

cellulose esters, especially with nitric acid and carboxylic acids, mixed cellulose esters, and cellulose ethers; natural polymers containing nitrogen, such as proteins and derivatives, including cross-linked or modified gelatins; natural hydrocarbon polymers, such as latex and rubber; synthetic polymers, such as vinyl polymers, including polyethylene, polypropylene, polystyrene, polyvinylchloride, polyvinylacetate and its partially hydrolyzed derivatives, polyacrylamides, polymethacrylates, copolymers and terpolymers of the above polycondensates, such as polyesters, polyamides, and other polymers, such as polyurethanes or polyepoxides; inorganic materials such as sulfates or carbonates of alkaline earth metals and magnesium, including barium sulfate, calcium sulfate, calcium carbonate, silicates of alkali and alkaline earth metals, aluminum and magnesium; and aluminum or silicon oxides or hydrates, such as clays, alumina, talc, kaolin, zeolite, silica gel, or glass (these materials may be used as filters with the above polymeric materials); and mixtures or copolymers of the above classes, such as graft copolymers obtained by initializing polymerization of synthetic polymers on a pre-existing natural polymer. All of these materials may be used in suitable shapes, such as films, sheets, tubes, particulates, or plates, or they may be coated onto, bonded, or laminated to appropriate inert carriers, such as paper, glass, plastic films, fabrics, or the like.

Nitrocellulose has excellent absorption and adsorption qualities for a wide variety of reagents including monoclonal antibodies. Nylon also possesses similar characteristics and also is suitable.

Preferred solid phase materials for flow-through assay devices include filter paper such as a porous fiberglass material or other fiber matrix materials. The thickness of such material is not critical and will be a matter of choice, largely based upon the properties of the sample or analyte being assayed, such as the fluidity of the sample.

Alternatively, the solid phase can constitute microparticles. Microparticles useful in the invention can be selected by one skilled in the art from any suitable type of particulate material and include those composed of polystyrene, polymethylacrylate, polypropylene, latex, polytetrafluoroethylene, polyacrylonitrile, polycarbonate, or similar materials. Further, the microparticles can be magnetic or paramagnetic

microparticles, so as to facilitate manipulation of the microparticle within a magnetic field.

Microparticles can be suspended in the mixture of soluble reagents and sample or can be retained and immobilized by a support material. In the latter case, the microparticles on or in the support material are not capable of substantial movement to positions elsewhere within the support material. Alternatively, the microparticles can be separated from suspension in the mixture of soluble reagents and sample by sedimentation or centrifugation. When the microparticles are magnetic or paramagnetic the microparticles can be separated from suspension in the mixture of soluble reagents and sample by a magnetic field.

The methods of the present invention can be adapted for use in systems that utilize microparticle technology including automated and semi-automated systems wherein the solid phase comprises a microparticle. Such systems include those described in pending U.S. App. No. 425,651 and U.S. Patent No. 5,089,424, which correspond to published EPO App. Nos. EP 0 425 633 and EP 0 424 634, respectively, and U.S. Patent No. 5,006,309.

In particular embodiments, the solid phase includes one or more electrodes. Capture agent(s) can be affixed, directly or indirectly, to the electrode(s). In one embodiment, for example, capture agents can be affixed to magnetic or paramagnetic microparticles, which are then positioned in the vicinity of the electrode surface using a magnet. Systems in which one or more electrodes serve as the solid phase are useful where detection is based on electrochemical interactions. Exemplary systems of this type are described, for example, in U.S. Patent No. 6,887,714 (issued May 3, 2005). The basic method is described further below with respect to electrochemical detection.

The capture agent can be attached to the solid phase by adsorption, where it is retained by hydrophobic forces. Alternatively, the surface of the solid phase can be activated by chemical processes that cause covalent linkage of the capture agent to the support.

To change or enhance the intrinsic charge of the solid phase, a charged substance can be coated directly onto the solid phase. Ion capture procedures for immobilizing an immobilizable reaction complex with a negatively charged polymer, described in U.S. App. No. 150,278, corresponding to EP Publication No. 0326100, and

U.S.App. No. 375,029 (EP Publication No. 0406473), can be employed according to the present invention to affect a fast solution-phase immunochemical reaction. In these procedures, an immobilizable immune complex is separated from the rest of the reaction mixture by ionic interactions between the negatively charged polyanion/immune complex and the previously treated, positively charged matrix and detected by using any of a number of signal-generating systems, including, e.g., chemiluminescent systems, as described in U.S. App. No. 921,979, corresponding to EPO Publication No. 0 273,115.

If the solid phase is silicon or glass, the surface must generally be activated prior to attaching the specific binding partner. Activated silane compounds such as triethoxy amino propyl silane (available from Sigma Chemical Co., St. Louis, Mo.), triethoxy vinyl silane (Aldrich Chemical Co., Milwaukee, Wis.), and (3-mercapto-propyl)-trimethoxy silane (Sigma Chemical Co., St. Louis, Mo.) can be used to introduce reactive groups such as amino-, vinyl, and thiol, respectively. Such activated surfaces can be used to link the capture directly (in the cases of amino or thiol), or the activated surface can be further reacted with linkers such as glutaraldehyde, bis (succinimidyl) suberate, SPPD 9 succinimidyl 3-[2-pyridyldithio] propionate), SMCC (succinimidyl-4-[Nmaleimidomethyl] cyclohexane-1-carboxylate), SIAB (succinimidyl [4iodoacetyl] aminobenzoate), and SMPB (succinimidyl 4-[1maleimidophenyl] butyrate) to separate the capture agent from the surface. Vinyl groups can be oxidized to provide a means for covalent attachment. Vinyl groups can also be used as an anchor for the polymerization of various polymers such as poly-acrylic acid, which can provide multiple attachment points for specific capture agents. Amino groups can be reacted with oxidized dextrans of various molecular weights to provide hydrophilic linkers of different size and capacity. Examples of oxidizable dextrans include Dextran T-40 (molecular weight 40,000 daltons), Dextran T-110 (molecular weight 110,000 daltons), Dextran T-500 (molecular weight 500,000 daltons), Dextran T-2M (molecular weight 2,000,000 daltons) (all of which are available from Pharmacia, Piscataway, N.J.), or Ficoll (molecular weight 70,000 daltons; available from Sigma Chemical Co., St. Louis, Mo.). Additionally, polyelectrolyte interactions can be used to immobilize a specific capture agent on a solid phase using techniques and chemistries described U.S.

App. No. 150,278, filed Jan. 29, 1988, and U.S. App. No. 375,029, filed Jul. 7, 1989, each of which is incorporated herein by reference.

Other considerations affecting the choice of solid phase include the ability to minimize non-specific binding of labeled entities and compatibility with the labeling system employed. For, example, solid phases used with fluorescent labels should have sufficiently low background fluorescence to allow signal detection.

Following attachment of a specific capture agent, the surface of the solid support may be further treated with materials such as serum, proteins, or other blocking agents to minimize non-specific binding.

Labeling Systems

As discussed above, many immunoassays according to the invention employ a labeled detection agent, such as a labeled species-specific antibody and a labeled endogenous cardiovascular antigen.

Detectable labels suitable for use in the detection agents of the present invention include any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical, or chemical means. Useful labels in the present invention include magnetic beads (e.g., Dynabeads^{TM}), fluorescent dyes (e.g., fluorescein, Texas Red, rhodamine, green fluorescent protein, and the like, see, e.g., Molecular Probes, Eugene, Oregon, USA), chemiluminescent compounds such as acridinium (e.g., acridinium-9-carboxamide), phenanthridinium, dioxetanes, luminol and the like, radiolabels (e.g., ^{3}H, ^{125}I, ^{35}S, ^{14}C, or ^{32}P), catalysts such as enzymes (e.g., horseradish peroxidase, alkaline phosphatase, beta-galactosidase and others commonly used in an ELISA), and colorimetric labels such as colloidal gold (e.g., gold particles in the 40-80 nm diameter size range scatter green light with high efficiency) or colored glass or plastic (e.g., polystyrene, polypropylene, latex, etc.) beads. Patents teaching the use of such labels include U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241.

The label can be attached to the detection agent prior to, or during, or after contact with the sample. So-called "direct labels" are detectable labels that are directly attached to or incorporated into detection agents prior to use in the assay. Direct labels

can be attached to or incorporated into detection agents by any of a number of means well known to those of skill in the art.

In contrast, so-called "indirect labels" typically bind to the detection agent at some point during the assay. Often, the indirect label binds to a moiety that is attached to or incorporated into the detection agent prior to use. Thus, for example, an antibody used as a detection agent (a "detection antibody") can be biotinylated before use in an assay. During the assay, an avidin-conjugated fluorophore can bind the biotin-bearing detection agent, to provide a label that is easily detected.

In another example of indirect labeling, polypeptides capable of specifically binding immunoglobulin constant regions, such as polypeptide A or polypeptide G, can also be used as labels for detection antibodies. These polypeptides are normal constituents of the cell walls of streptococcal bacteria. They exhibit a strong non-immunogenic reactivity with immunoglobulin constant regions from a variety of species (see, generally Kronval, et al. (1973) J. Immunol., 111: 1401-1406, and Akerstrom (1985) J. Immunol., 135: 2589-2542). Such polypeptides can thus be labeled and added to the assay mixture, where they will bind to the detection antibody, as well as to the species-specific antibody, labeling both and providing a composite signal attributable to analyte and autoantibody present in the sample.

Some labels useful in the invention may require the use of an indicator reagent to produce a detectable signal. In an ELISA, for example, an enzyme label (e.g., beta-galactosidase) will require the addition of a substrate (e.g., X-gal) to produce a detectable signal.

Exemplary Formats

Fluorescence Polarization Immunoassay (FPIA)

In an exemplary embodiment, a fluorescent label is employed in a fluorescence polarization immunoassay (FPIA) according to the invention. Generally, fluorescent polarization techniques are based on the principle that a fluorescent label, when excited by plane-polarized light of a characteristic wavelength, will emit light at another characteristic wavelength (i.e., fluorescence) that retains a degree of the polarization relative to the incident light that is inversely related to the rate of rotation of the label in a given medium. As a consequence of this property, a label with constrained rotation,

such as one bound to another solution component with a relatively lower rate of rotation, will retain a relatively greater degree of polarization of emitted light than when free in solution.

This technique can be employed in immunoassays according to the invention, for example, by selecting reagents such that binding of the fluorescently labeled entities forms a complex sufficiently different in size such that a change in the intensity light emitted in a given plane can be detected. For example, when a labeled endogenous cardiovascular antigen is bound by one or more autoantibodies, the resulting complex is sufficiently larger, and its rotation is sufficiently constrained, relative to the free labeled endogenous cardiovascular antigen that binding is easily detected.

Fluorophores useful in FPIA include fluorescein, aminofluorescein, carboxyfluorescein, and the like, preferably 5 and 6-aminomethylfluorescein, 5 and 6-aminofluorescein, 6-carboxyfluorescein, 5-carboxyfluorescein, thioureafluorescein, and methoxytriazinolyl-aminofluorescein, and similar fluorescent derivatives. Examples of commercially available automated instruments with which fluorescence polarization assays can be conducted include: the IMx® system, the TDx system, and TDxFLx® system (all available from Abbott Laboratories, Abbott Park, Ill.).

Scanning Probe Microscopy (SPM)

The use of scanning probe microscopy (SPM) for immunoassays also is a technology to which the immunoassay methods of the present invention are easily adaptable. In SPM, in particular in atomic force microscopy, the capture agent is affixed to a solid phase having a surface suitable for scanning. The capture agent can, for example, be adsorbed to a plastic or metal surface. Alternatively, the capture agent can be covalently attached to, e.g., derivatized plastic, metal, silicon, or glass according to methods known to those of ordinary skill in the art. Following attachment of the capture agent, the sample is contacted with the solid phase, and a scanning probe microscope is used to detect and quantify solid phase-affixed complexes. The use of SPM eliminates the need for labels which are typically employed in immunoassay systems. Such a system is described in U.S. App. No. 662,147, which is incorporated herein by reference.

MicroElectroMechanical Systems (MEMS)

Immunoassays according to the invention can also be carried out using a MicroElectroMechanical System (MEMS). MEMS are microscopic structures integrated onto silicon that combine mechanical, optical, and fluidic elements with electronics, allowing convenient detection of an analyte of interest. An exemplary MEMS device suitable for use in the invention is the Protiveris' multicantilever array. This array is based on chemo-mechanical actuation of specially designed silicon microcantilevers and subsequent optical detection of the microcantilever deflections. When coated on one side with a binding partner, a microcantilever will bend when it is exposed to a solution containing the complementary molecule. This bending is caused by the change in the surface energy due to the binding event. Optical detection of the degree of bending (deflection) allows measurement of the amount of complementary molecule bound to the microcantilever.

Electrochemical Detection Systems

In other embodiments, immunoassays according to the invention are carried out using electrochemical detection. A basic procedure for electrochemical detection has been described by Heineman and coworkers. This entailed immobilization of a primary antibody (Ab, rat-anti mouse IgG), followed by exposure to a sequence of solutions containing the antigen (Ag, mouse IgG), the secondary antibody conjugated to an enzyme label (AP-Ab, rat anti mouse IgG and alkaline phosphatase), and p-aminophenyl phosphate (PAPP). The AP converts PAPP to p-aminophenol (PAPR, the "R" is intended to distinguish the reduced form from the oxidized form, PAPO, the quinoneimine), which is electrochemically reversible at potentials that do not interfere with reduction of oxygen and water at pH 9.0, where AP exhibits optimum activity. PAPR does not cause electrode fouling, unlike phenol whose precursor, phenylphosphate, is often used as the enzyme substrate. Although PAPR undergoes air and light oxidation, these are easily prevented on small scales and short time frames. Picomole detection limits for PAPR and femtogram detection limits for IgG achieved in microelectrochemical immunoassays using PAPP volumes ranging from 20 μL to 360 mL have been reported previously. In capillary immunoassays with electrochemical detection, the lowest detection limit reported thus far is 3000 molecules of mouse IgG using a volume of 70 mL and a 30 minute or 25 minute assay time.

In an exemplary embodiment employing electrochemical detection, a capture agent according to the invention can be immobilized on the surface of an electrode (the "solid phase"). The electrode is then contacted with a sample from, e.g., a human. Any anti-endogenous cardiovascular antigen antibodies in the sample bind to the capture agent to form a solid phase-affixed complex. Anti-human antibody, which is labeled with AP, for example, binds to autoantibodies in the complex, thereby becoming immobilized on the surface of the electrode. The addition of PAPP, results in its conversion by AP to PAPR, which is then detected.

Various electrochemical detection systems are described in U.S. Patent Nos. 7,045,364 (issued May 16, 2006; incorporated herein by reference), 7,045,310 (issued May 16, 2006; incorporated herein by reference), 6,887,714 (issued May 3, 2005; incorporated herein by reference), 6,682,648 (issued January 27, 2004; incorporated herein by reference); 6,670,115 (issued December 30, 2003; incorporated herein by reference).

The present invention is for example applicable to point of care assay systems, including Abbott Laboratories' commercial Point of Care (i-STAT®) electrochemical immunoassay system which performs sandwich immunoassays for several cardiac markers, including TnI, CKMB and BNP. Immunosensors and methods of manufacturing and operating them in single-use test devices are described, for example, in U.S. Patent No. 5,063,081 and published U.S. Patent Application Nos. US 20030170881, US 20040018577, US 20050054078, and US 20060160164, each of which is incorporated herein by reference for their teachings regarding same.

Additionally, it goes without saying that any of the exemplary formats herein, and any assay or kit according to the invention can be adapted or optimized for use in automated and semi-automated systems (including those in which there is a solid phase comprising a microparticle), as described, e.g., in U.S. Patent Nos. 5,089,424 and 5,006,309, and as, e.g., commercially marketed by Abbott Laboratories (Abbott Park, IL), including but not limited to Abbott's ARCHITECT®, AxSYM®, IMx®, ABBOTT PRISM®, and Quantum II platforms, as well as other platforms.

Multiplex Formats (Exemplary Panel)

In particular embodiments, useful, for example, for simultaneously assaying multiple analytes in one sample, the solid phase can include a plurality of different capture agents, including one that captures autoantibodies to endogenous cardiovascular antigen. Thus, for example, the solid phase can have affixed thereon a plurality of antigens, wherein each is intended to test for the presence of different autoantibodies in the sample. In an exemplary embodiment, the solid phase can consist of a plurality of different regions on a surface, wherein each region has a particular endogenous cardiovascular antigen affixed therein. Optionally such a solid phase comprises a so-called "panel", such that the invention optionally provides a rapid multiple panel assay containing endogenous cardiac antigens for the detection of autoantibodies.

In one embodiment, the endogenous cardiac antigens comprising the panel are present on an array. The term "array" refers to a solid-phase or gel-like carrier upon which at least two compounds are attached or bound in one-, two- or three-dimensional arrangement. Such arrays (including "chips", antibody arrays, and the like) are generally known to the person skilled in the art and typically generated on glass microscope slides, specially coated glass slides such as polycation-, nitrocellulose- or biotin-coated slides, cover slips, and membranes such as, for example, membranes based on nitrocellulose or nylon. However, it is not strictly necessary that the members of the panel constitute an array. For instance, it is possible that assays can be run in series, such that the panel assay comprises collection of independent assay results that separately and independently assess autoantibodies to endogenous cardiac antigens.

Multiplex formats can, but need not, employ a plurality of labels, wherein each label is used for the detection of autoantibodies reactive with a particular antigen. For example, multiple, different autoantibodies can be detected without using a plurality of labels where a plurality of capture agents, such as antigens, are affixed to the solid phase at different known locations, based on specificity. Because the specificity of the capture agent at each location is known, the detection of a signal at a particular location can be associated with the presence of autoantibodies bound at that location. Examples of this format include microfluidic devices and capillary arrays, containing different capture agents at different locations along a channel or capillary, respectively, and microarrays, which typically contain different capture agents arranged in a matrix of

spots ("target elements") on a surface of a solid support. In particular embodiments, each different capture agent can be affixed to a different electrode, which can, for example, be formed on a surface of a solid support, in a channel of a microfluidic device, or in a capillary.

Automated Instrumentation

Optionally the immunoassays as described herein can be used in kits for commercial platform immunoassays (e.g., assays on Abbott's PRISM®, AxSYM®, ARCHITECT® and/or EIA (Bead) platforms, as well as in other commercial and/or in vitro diagnostic assays.

Test Kits

The invention also provides test kits for assaying samples for autoantibodies to endogenous cardiovascular antigens. Test kits according to the invention include one or more reagents useful for practicing one or more immunoassays according to the invention. A test kit generally includes a package with one or more containers holding the reagents, as one or more separate compositions or, optionally, as admixture where the compatibility of the reagents will allow. The test kit can also include other material(s), which may be desirable from a user standpoint, such as a buffer(s), a diluent(s), a standard(s), and/or any other material useful in sample processing, washing, or conducting any other step of the assay.

In certain embodiments, a test kit includes a humanized monoclonal antibody, wherein the humanized monoclonal antibody is specific for an endogenous cardiovascular antigen. This component can be used as a positive control in immunoassays according to the invention. If desired, this component can be included in the test kit in multiple concentrations to facilitate the generation of a standard curve to which the signal detected in the test sample can be compared. Alternatively, a standard curve can be generated by preparing dilutions of a single humanized monoclonal antibody solution provided in the kit.

Kits according to the invention can include a solid phase and a capture agent affixed to the solid phase, wherein the capture agent is selected from the group consisting of an endogenous cardiovascular antigen and a species-specific antibody, wherein the species-specific antibody is specific for the species from which the sample is to be obtained. Where such kits are to be employed for conducting sandwich immunoassays, the kits can additionally include a labeled detection agent. In such embodiments, if the capture agent is an endogenous cardiovascular antigen, the detection agent can be a species-specific antibody. If the capture agent is a species-specific antibody, an endogenous cardiovascular antigen can be used as the detection agent. In particular embodiments, the species-specific antibody is a human-specific antibody.

Test kits according to the invention can also include a labeled non-human monoclonal antibody that is specific for an endogenous cardiovascular antigen. This component is useful as a control for confirming that any endogenous cardiovascular antigen employed is capable of binding antibody.

In certain embodiments, the test kit includes at least one direct label, such as acridinium-9-carboxamide. Test kits according to the invention can also include at least one indirect label. If the label employed generally requires an indicator reagent to produce a detectable signal, the test kit preferably includes one or more suitable indicator reagents.

In exemplary embodiments, the solid phase includes one or more microparticles or electrodes. Test kits designed for multiplex assays conveniently contain one or more solid phases including a plurality of antigens that are specific for a plurality of different autoantibodies. Thus, for example, a test kit designed for multiplex electrochemical immunoassays can contain a solid phase including a plurality of electrodes, with each electrode bearing a different antigen.

Test kits according to the invention preferably include instructions for carrying out one or more of the immunoassays of the invention. Instructions included in kits of the invention can be affixed to packaging material or can be included as a package insert. While the instructions are typically written or printed materials they are not limited to such. Any medium capable of storing such instructions and communicating them to an end user is contemplated by this invention. Such media include, but are not

limited to, electronic storage media (e.g., magnetic discs, tapes, cartridges, chips), optical media (e.g., CD ROM), and the like. As used herein, the term "instructions" can include the address of an internet site that provides the instructions.

The disclosure of all patents, publications, including published patent applications, and database entries referenced in this specification are specifically incorporated by reference in their entirety to the same extent as if each such individual patent, publication, and database entry were specifically and individually indicated to be incorporated by reference.

Although the invention has been described with reference to certain specific embodiments, various modifications thereof will be apparent to those skilled in the art without departing from the spirit and scope of the invention. All such modifications as would be apparent to one skilled in the art are intended to be included within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A method for assessing the risk of whether a subject has or might develop a cardiovascular autoimmune disease, wherein said method comprises:

(a) obtaining a sample from said subject;

(b) measuring the levels of autoantibodies to each of a plurality of different endogenous cardiovascular antigens in said subject's sample;

(c) comparing said levels with the level of the same autoantibodies measured in samples from reference subjects with clinically normal cardiovascular function, or from reference subjects having cardiovascular disease, and

(d) identifying said risk that said subject has or might develop a cardiovascular autoimmune disease based on the comparison in step (c).

2. The method of claim 1, wherein said risk is present when levels of autoantibodies to at least two of said different endogenous cardiovascular antigens:

(i) are elevated in said subject's sample as compared to in samples from reference subjects with clinically normal cardiovascular function, and/or

(ii) are elevated in or at about the same level in said subject's sample as compared to in samples from reference subjects having cardiovascular disease.

3. The method of claim 1, wherein said risk is further increased when said subject either has an autoimmune disease, or is a first-degree relative of an individual having an autoimmune disease

4. The method of claim 1, wherein said levels of autoantibodies to at least two of said different endogenous cardiovascular antigens are elevated due to the presence of cardiovascular autoimmune disease.

5. The method of claim 1, wherein said levels of autoantibodies to at least two of said different endogenous cardiovascular antigens are elevated due to an increased risk of developing cardiovascular autoimmune disease.

6. The method of claim 1, wherein said subject's sample comprises peripheral blood, serum, plasma, cerebrospinal fluid, urine, or other body fluid sample.

7. The method of claim 1, wherein said cardiovascular autoimmune disease is a disease, disorder or condition selected from the group consisting of myocarditis, cardiomyopathy, and ischemic heart disease.

8. The method of claim 1, wherein said plurality comprises two or more.

9. The method of claim 1, wherein said plurality comprises between two and ten.

10. The method of claim 1, wherein said plurality comprises between five and ten.

11. The method of claim 1, wherein said plurality comprises a number selected from the group consisting of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 15, 17, 18, 19, and 20.

12. The method of any of claims 1 through 11, wherein said different endogenous cardiovascular antigens are selected from the group consisting of α1-adrenoceptor, angiotensin-1 receptor, annexin V, brain natriuretic peptide, cardiac troponins, myosin, tropomyosin, cytoplasmic neutrophils, endothelial receptor of protein C, Factor VIII, grehlin, halogenated protein, nitrated protein, heat shock proteins, myeloperoxidase, placental growth factor, phospholipids, proteinase-3, prothrombin, Purkinje fibers, sarcolemmal Na-K-ATPase, β1-adrenoceptor, β2-adrenoceptor, and tissue-type plasminogen activator.

13. The method of claim 12, wherein said different endogenous cardiovascular antigens further comprise an antigen selected from the group consisting of cardiolipin, oxidized LDL, and β-2-glycoprotein-1.

14. A method for identifying a patient with cardiovascular disease as eligible to receive autoimmune therapy, wherein said method comprises:

(a) obtaining a sample from said cardiovascular disease patient;

(b) measuring the levels of autoantibodies to each of a plurality of different endogenous cardiovascular antigens in said subject's sample;

(c) comparing said levels with the level of the same autoantibodies measured in samples from reference subjects with clinically normal cardiovascular function, or from reference subjects having autoimmune disease; and

(d) identifying said cardiovascular disease patient as eligible to receive autoimmune therapy based on the comparison in step (c).

15. The method of claim 14, wherein said patient is eligible to receive autoimmune therapy when levels of autoantibodies to at least two of said different endogenous cardiovascular antigens:

(i) are elevated in said cardiovascular disease patient's sample as compared to in samples from reference subjects with clinically normal cardiovascular function, and/or

(ii) are elevated in or at about the same in said cardiovascular disease patient's sample as compared to in samples from reference subjects having autoimmune disease.

16. The method of claim 14, wherein said cardiovascular disease patient further is eligible to receive autoimmune therapy when said subject either has an autoimmune disease, or is a first-degree relative of an individual having an autoimmune disease

17. The method of claim 14, wherein said levels of autoantibodies to at least two of said different endogenous cardiovascular antigens are elevated due to the presence of cardiovascular autoimmune disease.

18. The method of claim 14, wherein said levels of autoantibodies to at least two of said different endogenous cardiovascular antigens are elevated due to an increased risk of developing cardiovascular autoimmune disease.

19. The method of claim 14, wherein said cardiovascular disease patient's sample comprises peripheral blood, serum, plasma, cerebrospinal fluid, urine, or other body fluid sample.

20. The method of claim 14, wherein said cardiovascular autoimmune disease is a disease, disorder or condition selected from the group consisting of myocarditis, cardiomyopathy, and ischemic heart disease.

21. The method of claim 14, wherein said plurality comprises two or more.

22. The method of claim 14, wherein said plurality comprises between two and ten.

23. The method of claim 14, wherein said plurality comprises between five and ten.

24. The method of claim 14, wherein said plurality comprises a number selected from the group consisting of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 15, 17, 18, 19, and 20.

25. The method of any of claims 14 to 24, wherein said different endogenous cardiovascular antigens are selected from the group consisting of α1-adrenoceptor, angiotensin-1 receptor, annexin V, brain natriuretic peptide, cardiac troponins, myosin, tropomyosin, cytoplasmic neutrophils, endothelial receptor of protein C, Factor VIII, grehlin, halogenated protein, nitrated protein, heat shock proteins, myeloperoxidase, placental growth factor, phospholipids, proteinase-3, prothrombin, Purkinje fibers, sarcolemmal Na-K-ATPase, β1-adrenoceptor, β2-adrenoceptor, and tissue-type plasminogen activator.

26. The method of claim 25, wherein said different endogenous cardiovascular antigens further comprise an antigen selected from the group consisting of cardiolipin, oxidized LDL, and β-2-glycoprotein-1.

27. A method of identifying a subject having, or at risk for developing, a cardiovascular autoimmune disease, the method comprising assaying a sample from the subject for the levels of autoantibodies to each of a plurality of different endogenous cardiovascular antigens, wherein the subject has an autoimmune disease, or the subject is a first-degree relative of an individual having an autoimmune disease, and wherein the presence of elevated levels of autoantibodies to at least two of said different endogenous cardiovascular antigens indicates the presence of, or risk of, said cardiovascular autoimmune disease.

28. A test kit for assaying a sample for autoantibodies to at least two different endogenous cardiovascular antigens, the test kit comprising means for the detection of levels of autoantibodies to each of a plurality of different endogenous cardiovascular antigens.

29. The test kit of claim 28, wherein said test kit additionally comprises a solid phase and a capture agent affixed to said solid phase, wherein the capture agent is a peptide having a sequence that corresponds to a portion or the entirety of the amino acid sequence of the endogenous cardiovascular antigens selected from the group consisting of α1-adrenoceptor, angiotensin-1 receptor, annexin V, brain natriuretic peptide, cardiac troponins, myosin, tropomyosin, cytoplasmic neutrophils, endothelial receptor of protein C, Factor VIII, grehlin, halogenated protein, nitrated protein, heat shock proteins, myeloperoxidase, placental growth factor, phospholipids, proteinase-3, prothrombin, Purkinje fibers, sarcolemmal Na-K-ATPase, β1-adrenoceptor, β2-adrenoceptor, and tissue-type plasminogen activator.

30. The test kit of claim 29, wherein said different endogenous cardiovascular antigens further comprise an antigen selected from the group consisting of cardiolipin, oxidized LDL, and β-2-glycoprotein-1.

31. The test kit of any of claims 28 through 30, wherein the test kit additionally comprises a labeled detection agent, comprising a species-specific antibody.

32. The test kit of claim 31, additionally comprising an indicator reagent that interacts with said label to produce a detectable signal.

33. The test kit of claim 29 or 30, wherein the solid phase comprises a microplate.

34. The test kit of claim 29 or 30, wherein the solid phase comprises a microparticle.

35. The test kit of claim 29 or 30, wherein the solid phase comprises an electrode.

36. A method for assessing the risk of whether a subject has or might develop a cardiovascular autoimmune disease, wherein said method comprises:

(a) obtaining a sample from said subject;

(b) measuring the levels of one or more endogenous cardiovascular antigens in said subject's sample;

(c) measuring the levels of autoantibodies to each of a plurality of different endogenous cardiovascular antigens in said subject's sample;

(d) comparing said levels of said one or more endogenous cardiovascular antigens with the level of the same endogenous cardiovascular antigen measured in samples from reference subjects with clinically normal cardiovascular function, in samples from reference subjects having cardiovascular disease, or in said subject's sample obtained at an earlier time;

(e) comparing said levels of autoantibodies with the level of the same autoantibody measured in samples from reference subjects with clinically normal

cardiovascular function, in samples from reference subjects having cardiovascular disease, or in said subject's sample obtained at an earlier time; and

(f) identifying said risk that said subject has or might develop a cardiovascular autoimmune disease based on the comparison in steps (d) and (e).

37. The method of claim 36, wherein said risk is present when:

(i) in step (d) the levels of said one or more endogenous cardiovascular antigens are altered in said subject's sample as compared to in samples from reference subjects with clinically normal cardiovascular function, and/or are altered in or at about the same level in said subject's sample as compared to in said subject's sample obtained at an earlier time; and/or

(ii) in step (e) the levels of said autoantibodies to at least two of said different endogenous cardiovascular antigens are elevated in said subject's sample as compared to in samples from reference subjects with clinically normal cardiovascular function, and/or are elevated in or at about the same level in said subject's sample as compared to in samples from reference subjects having cardiovascular disease.

38. The method of claim 36 or 37, wherein the measuring of step (b) and the measuring of step (c) are done simultaneously.

39. The method of claim 36 or 37, wherein the measuring of step (b) and the measuring of step (c) are done sequentially, in any order.

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摘要(译)

本文提供的是诊断测试，使用方法和用于评估和管理心血管自身免疫疾病和心血管自身免疫疾病风险的试剂盒。本发明的测定方法尤其可用于鉴定患有心血管疾病，自身免疫疾病或与患有自身免疫疾病的个体相关的受试者的心血管自身免疫疾病或其风险。该方法可用于测试表现出心血管疾病症状的受试者，以及显然健康且尚未表现出心血管疾病症状但可能随时间推移的受试者。在一个实施方案中，本发明还提供了确定患有心血管疾病或有患心血管疾病风险的受试者是否是免疫抑制疗法或免疫吸收疗法的候选者的方法。本发明还提供了可用于实施本发明方法的试剂盒和试剂盒组分。