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(54) **BIOCHEMICAL MARKERS FOR CVD RISK ASSESSMENT**

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(57) **ABSTRACT**

A method of diagnosis of cardiovascular disease (CVD) an immunoassay to measure aggrecan fragments in said sample, and association of an elevation above a normal level with the presence of CVD, is conducted by contacting aggrecan fragments in said sample with an first antibody reactive with an N-terminal first epitope formed by cleavage of aggrecan by a proteinase and with a second antibody reactive with a second aggrecan epitope which is present in aggrecan at a location in the C-terminal direction from the location of said N-terminal epitope, and measuring the extent of simultaneous binding of both antibodies.

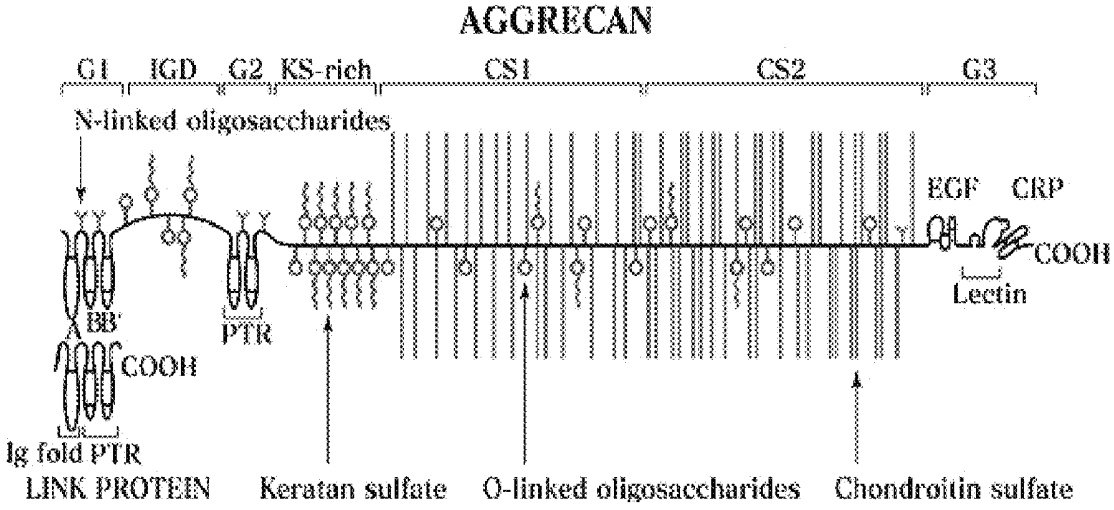
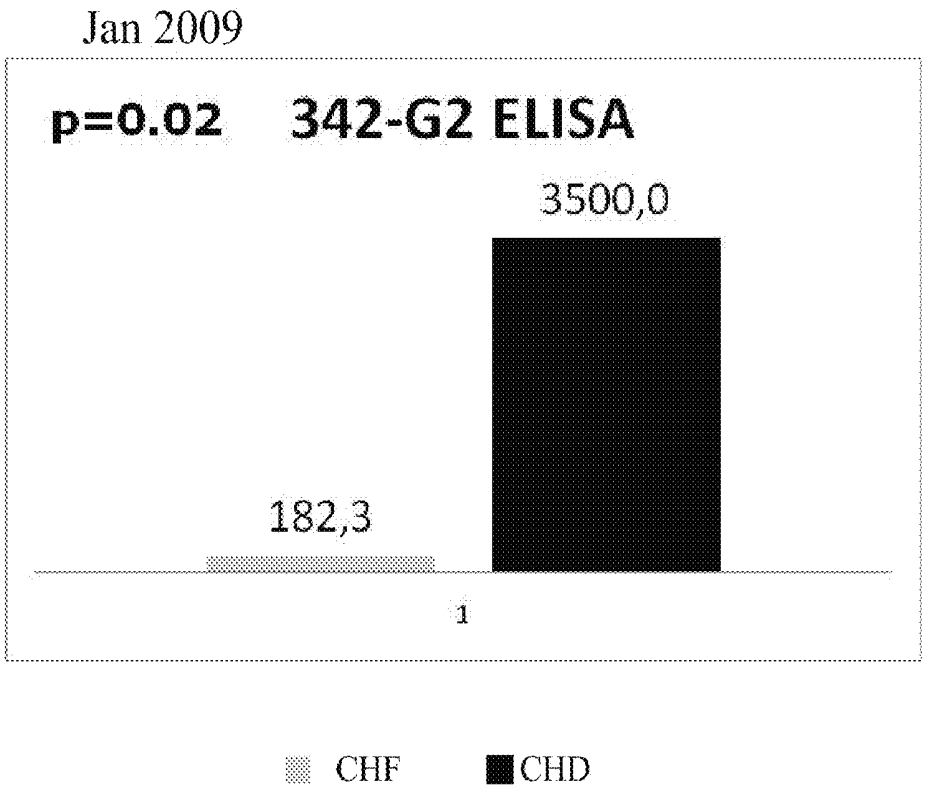

AGGRECAN
G1
IGD
G2
KS-rich
CS1
CS2
G3
N-linked oligosaccharides
EGF
CRP
COOH
Lectin
BB'
PTR
COOH
Ig fold PTR
LINK PROTEIN
Keratan sulfate
O-linked oligosaccharides
Chondroitin sulfate

Figure 1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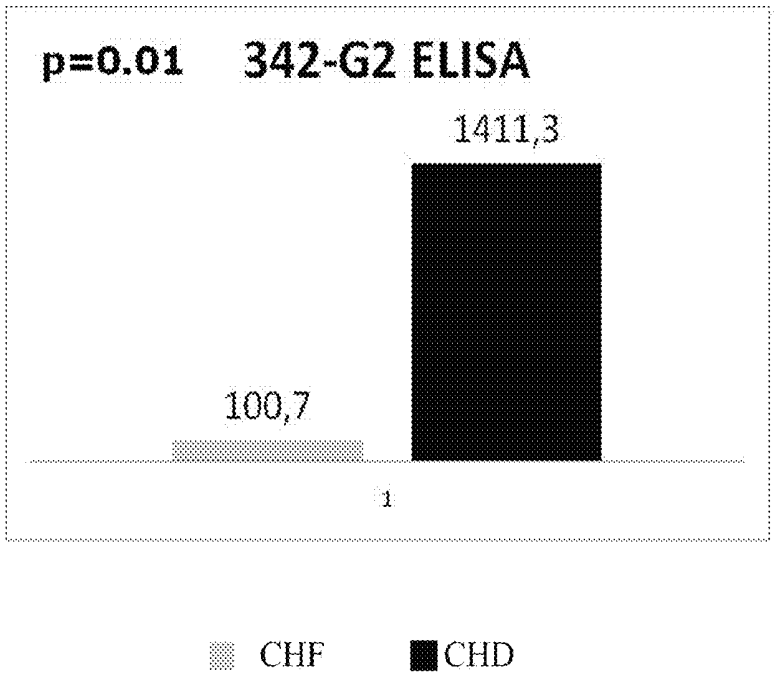
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CHF
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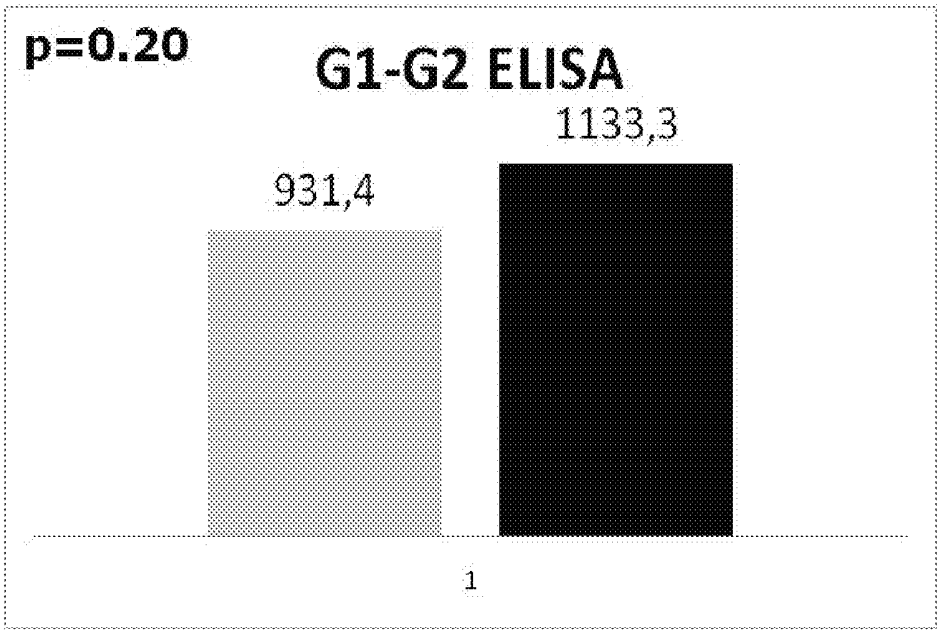
Figure 2A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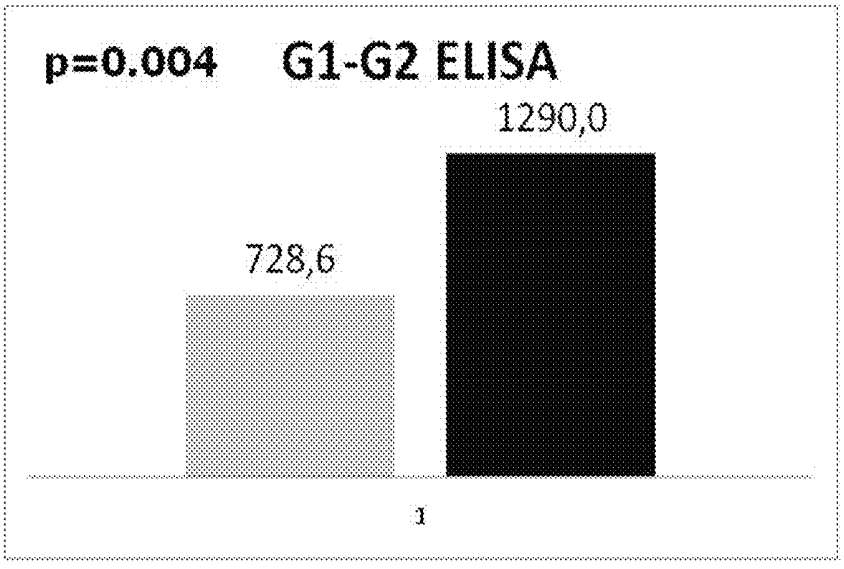
931,4
1
CHF
CHD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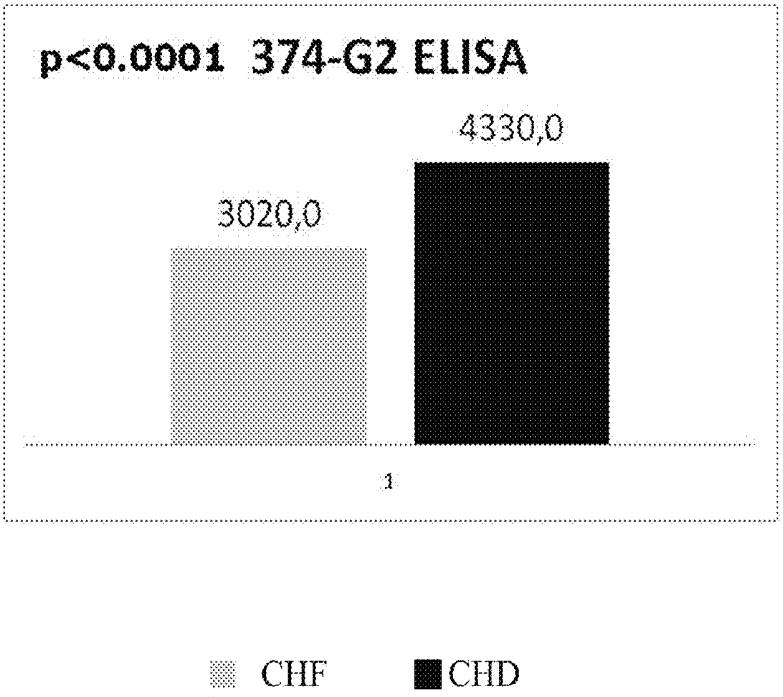
CHF
CHD

Figure 2B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3020,0
1
CHF
CHD

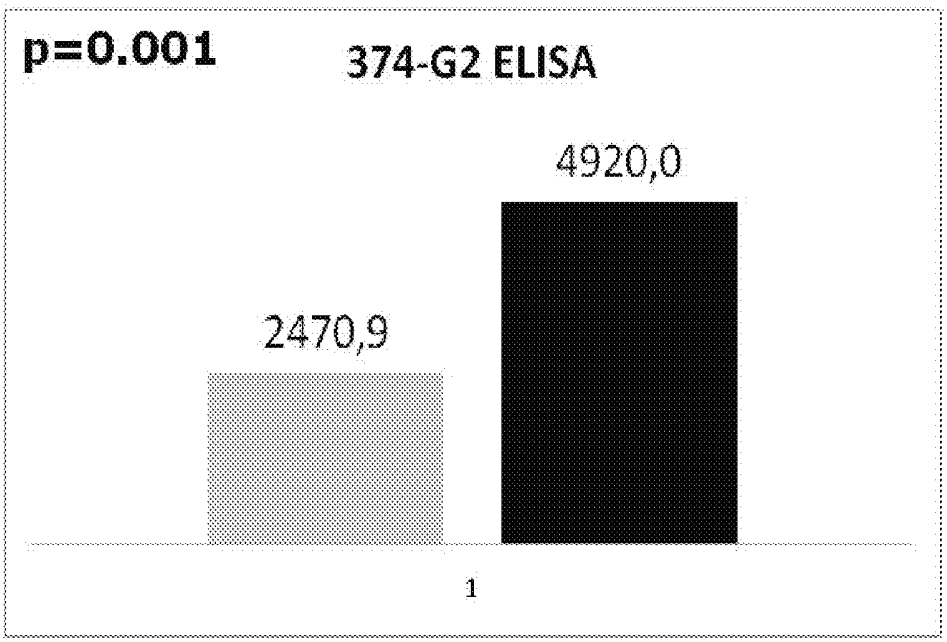
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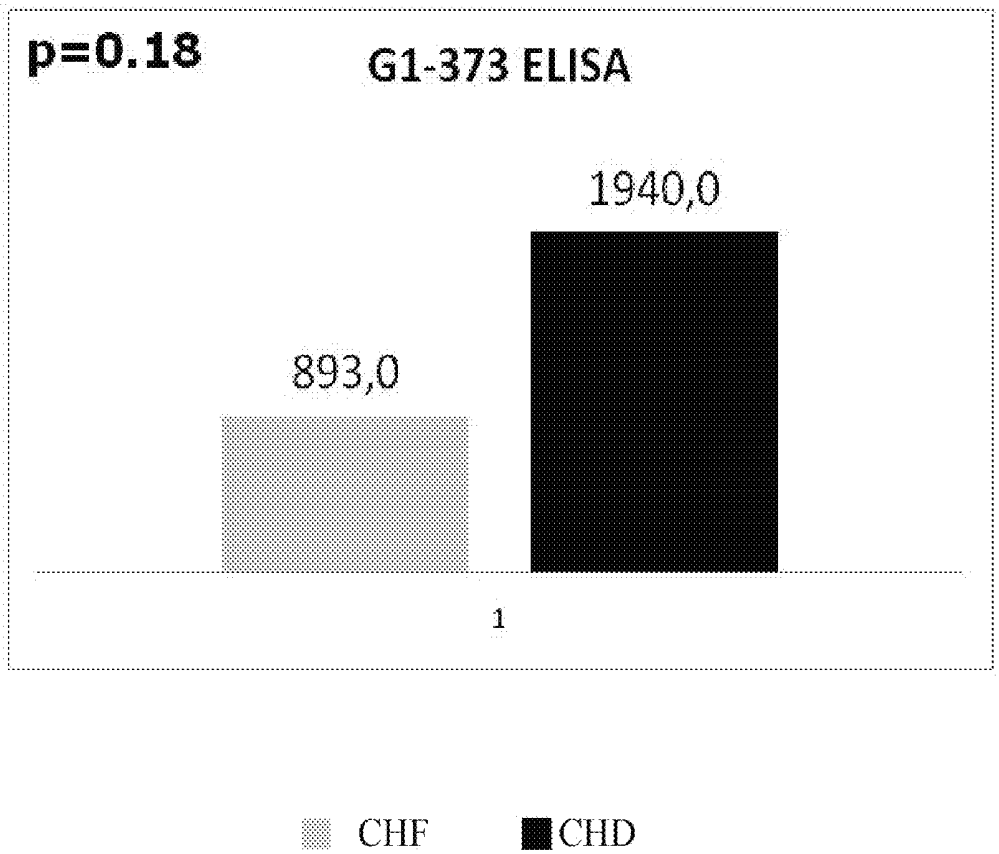
Figure 2C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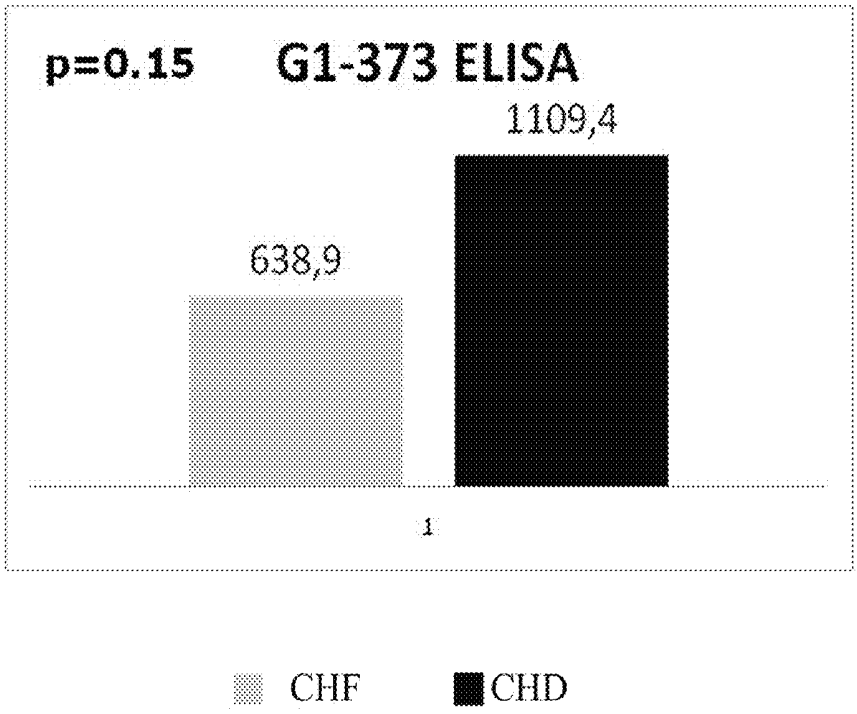
p=0.15
G1-373 ELISA
1109,4
638,9
1
CHF
CHD

Figure 2D

BIOCHEMICAL MARKERS FOR CVD RISK ASSESSMENT

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. provisional application Ser. No. 61/268,224, filed Jun. 9, 2009, the entire disclosure of which is incorporated herein by this reference.

SEQUENCE LISTING

[0002] A listing of amino acid sequences appearing herein is filed herewith.

BACKGROUND OF THE INVENTION

[0003] The present invention relates to assays for detection of biochemical markers valuable for diagnostic purposes in cardiovascular disease and prognosis of disease development, including biochemical markers indicative of the risk of cardiovascular events resulting from atherosclerotic development and plaque instability. In particular, the present invention relates to the detection of aggrecan and its fragments.

[0004] Worldwide, cardiovascular disease (CVD) is the leading cause of morbidity and mortality. At present, there are no effective and non-invasive diagnostic methods that allow for diagnosis and classification of patients into different risk-groups and for the diagnosis of low risk patients. Diagnostic and prognostic tools are composed mainly of multivariate analysis of simple markers, such as age, smoking and various lipid and lipoprotein concentrations.

[0005] CVD covers several clinical syndromes, primarily, angina pectoris, myocardial infarction (coronary thrombosis) and stroke. All of these syndromes are usually the sequelae of complicated atherosclerosis.

[0006] Atherosclerosis begins with intimal thickening in childhood and progresses to fatty streaks in the intima of arteries—these lesions are characterized as type I and II, respectively. Fatty streaks are the earliest macroscopically visible lesions in the development of atherosclerosis and occur among almost all human beings of all races and societies. In the non pathogenic state, endothelial cells (EC) resist adhesive interactions with leukocytes. However, the actions of proinflammatory cytokines and accumulated oxidized lipoprotein in the arterial wall during atherogenesis, initiate expression of adhesion molecules, such as intercellular adhesion molecules (ICAM)-1 and vascular cell adhesion molecules (VCAM)-1, on the surface of aortic ECs. This allows for capturing and transmigration of leukocytes through the endothelial surface, into the intimal part of the vessel wall. The development of plaques involves an increasing number of smooth muscle cells (SMC) that undergo displacement and apoptosis, which results in increased matrix turnover. The impaired collagen synthesis can result in a weakened fibrous cap and an atherosclerotic plaque that is more prone to rupture; however, most investigators believe that the actions of a proteolytic enzymes such as matrix metallo-proteases (MMPs) and other proteases importantly contribute to the risk of plaque rupture (Clarkson and Kaplan 509-28).

[0007] Plaques are divisible into two different types: 'vulnerable' and 'stabilized' plaques. However, for detailed histological analyses and molecular understanding, a more detailed classification is often used. There are three major stages in development of plaque: initiation, fatty streaks and the complex/advanced plaque (Stary H. C.).

[0008] Atherosclerotic plaques develop within the intima of arteries, and may be classified depending on their composition and structure. This classification divides lesions into eight types (Stary H. C.):

[0009] I. Macrophages loaded with and enlarged by lipid droplets (macrophage foam cells) are increased in the intima.

[0010] II. Macrophage foam cells accumulate in the deep part of the proteoglycan layer along with lipid droplets within the intimal SMC. The layers of foam cells are visible as fatty streaks. In type II lesions monocytes penetrate the endothelial lining by monocyte chemo attractant proteins (mainly MCP-1), which are over expressed in human atheroma. The early types of lesion (type I and II) can start in infancy and do not necessarily lead to plaque rupture. Furthermore, the development of atherosclerosis may end after the formation of type III lesion, and the formation of plaque is not predictable (Stary H. C.).

[0011] III. The type III lesion is determined as the intermediate lesion between the fatty streaks (type II) and the atheroma (type IV). These lesions contain pools of extracellular lipid and thereby expand the spaces between the normally closely adjoining SMCs of the deep musculo-elastic layer of the intima. The pools of material may replace proteoglycans and collagen fibres that normally reside here, but this occurs with little impact at this stage of atherogenesis.

[0012] IV. The atheroma is the first clinical sign of atherosclerosis. Displacement of SMCs in the intima of arteries by accumulating extracellular pools of lipids and disruption of the intimal architecture is a hallmark of a type IV lesion. The formation of the lipid cores is the end result of this SMC displacement. Formation of a lipid core accounts for the increased wall thickening. The lipid core is a large and well delineated region of the deep intima where the normal structural elements of this part of the arterial wall have been replaced by densely packed foam cell remnants, free lipids droplets, cholesterol crystals and calcium particles. SMCs normally resident in this area are decreased or completely absent at this stage of atherosclerosis progression. Any remnant SMCs become widely dispersed and have developed elongated cell bodies and very often unusually thick basement membranes. At this stage, the development of a layer overlying the lipid core begins. This layer consists of collagen and proteoglycan-rich intercellular matrix, SMCs with and without lipid droplets, macrophages, and foam cells.

[0013] V. The response to type IV lesion is the formation of a reparative fibrous tissue matrix, forming a fibrous "cap". Typically, these lesions will consist of layers of lipid cores and reparative tissue irregularly stacked on top of each other. Events such as hematoma and thrombus formation may additionally complicate these types of lesions. If not fatal, these lesion complications are integrated into the lesion and overgrown by a thin layer of reparative matrix tissue, consisting of collagens and proteoglycans. The content of extracellular matrix proteins collagen and proteoglycans increases in the atherosclerotic plaque during formation of the cap.

[0014] VI. The defects of the endothelium such as fissures, erosions, ulcerations, hematoma, thrombus,

haemorrhage can if combined lead to more complicated lesion type designated type VI lesion.

[0015] VII. The lesion is often referred to as calcified lesion, where more than 50% of the lesion consists of mineral. In addition to calcifications, these lesions contain abundance of reparative fibrous connective tissue. When the SMCs trapped in this undergo apoptosis and disintegrate; their mineralized organelles become a part of the calcification.

[0016] VIII. The fibrotic lesion follows the calcific lesion. The fibrotic lesion may consist entirely of collagen and no lipid. (Stary H. C.)

[0017] Cardiovascular events are often the result of plaque rupture, in which inflammation and the release of proteases weaken the shoulder regions of the fibrous cap and allow the fatty materials in the plaque to come into contact with the blood precipitating a mural thrombus (Clarkson and Kaplan). Thinning of the fibrous cap by increased protease activity in the combination with decreased matrix production, is considered a hallmark of plaque instability increasing the risk of rupture. Vulnerability of plaques and their risk of rupture is an area of clinical interest. Definition of a vulnerable plaque (VP) is not standardized, but there is a general agreement stating existence of three histological hallmarks compared to stable plaque:

[0018] 1) A larger lipid core (>40 percent of total lesion).

[0019] 2) A thinner fibrous cap (65-150 micrometers).

[0020] 3) Large amount of acute inflammatory cells.

Major criteria for defining VP include: active inflammation (presence of monocytes, macrophages and T cells), thin cap with large lipid core, endothelial denudation with superficial platelet aggregation, fissured plaque, and >90% stenosis of the artery. Other minor criteria include: superficial calcified nodule, intraplaque haemorrhage, endothelial dysfunction, and outward remodelling (Shin, Edelberg, and Hong).

[0021] Plaque complications, instability and rupture may be inhibited by medical treatment and/or lifestyle modification. In some cases, however, more invasive methods may be needed, i.e. angioplasty or bypass surgery.

[0022] Presently, diagnostic tools are based on either static image analyses still under development or low-technology methods such as systolic and diastolic blood pressure levels related to the risk of CVD. The field has devoted much attention to the development of multivariate analysis that may better identify patients at high risk. One such model is the SCORE-model (Systematic Coronary Risk Evaluation model). In 1994, with a revision in 2003, The European Atherosclerosis Society, The European Society of Cardiology and The European Society of Hypertension issued a set of recommendations regarding prevention of coronary heart diseases. This guideline is based on several assessment techniques, which have been developed to assess the risk of CVD in asymptomatic subjects, i.e. identification of asymptomatic high-risk patients. The SCORE-model integrates gender, age, smoking, systolic blood pressure and either total cholesterol or the cholesterol/HDL ratio as risk factors (Graham et al.).

[0023] In order to make a more detailed diagnosis, the SCORE model is not sufficient and imaging techniques are used. Imaging methods are therefore used mostly on patients in the high-risk group or during research.

[0024] A range of different biochemical markers have been suggested as markers of cardiovascular events. Wang et al (2006) have measured 10 different biochemical markers in 3200 patients participating in the Framingham study, described in Table 1. The conclusion was that the measurement of 10 biochemical markers only contributes moderately to diagnosis over and above standard risk factors. Of the 10 biochemical markers, B-type natriuretic peptide level, C-reactive protein level and the urinary albumin-to-creatinine ratio showed the best correlation between marker and death/cardiovascular events (Wang et al.).

Proteoglycans as Matrix Components

[0025] Proteoglycans (PG) are polysaccharide-protein macromolecules localized predominately in the intercellular matrix of vessel wall (Salisbury and Wagner 1981). PGs are macromolecules characterized by the presence of one, or more, long un-branched and highly polyanionic sugar side chains called GAGs, covalently attached to a core protein through a link region. The repeating unit of the GAG consists of an amino sugar, either N-acetyl-glucosamine (GlcNAc) or N-acetyl-galactosamine (GalNAc), and a hexuronic acid, either glucouronic acid (GlcA) or iduronic acid (IdoA). One or both of the sugars in the repeating unit contain one or more sulfate groups (Rodriguez-Lee 2007). In addition to the GAG chains, most core proteins carry N- and/or O-linked oligosaccharides.

Classification and Nomenclature of PGs

[0026] PGs are a very heterogeneous group of macromolecules. A single type of core protein can vary in the number and type of attached GAG chains. The length of the chains and the arrangement of the sulfated residues along the chains vary also.

[0027] Four main classes of GAGs are distinguished according to the structure of the repeating disaccharide unit: chondroitin sulfate (CS) and dermatan sulfate (DS), heparin sulfate (HS) and heparin, hyaluronan, and keratin sulfate (KS).

[0028] Chondroitin/dermatan sulfate PGs (versican, aggrecan, neurocan, and brevican) belong to the family of hyaluronan-binding proteoglycans. This gene family is collectively termed hyalectans. Proteoglycans (PGs) are macromolecules distributed almost everywhere in the human body. The structure and size of PGs vary extremely. The basic structure of all PGs includes a core protein and at least one, but often many carbohydrate chains-glycosaminoglycans (GAGs). PGs can be found intracellularly, on the surface of cells, and in the extracellular matrix. Each family member has a characteristic distribution, with aggrecan prominent in cartilage, neurocan and brevican prominent in the central nervous system, and versican present in a variety of soft tissues, including arterial walls.

[0029] Aggrecan is heavily glycosylated and comprises more than 2000 amino-acid residues. Aggrecan is structurally organized in three distinct domains: G1, G2 and G3 (FIG. **1**). Interspaced between the G2 and G3 domain, and to a lesser extent between the G1 and G2 domains are long stretches of heavily glycosylated regions, containing the negatively charged chondroitin sulphate and keratan sulphate oligosaccharide structures (Fosang 1995). The amino acid sequence of human aggrecan is given in SEQ ID NO 1.

Protease Profiles

[0030] Proteases hydrolyse peptide bonds and are responsible for the degradation of extracellular matrix proteins such as collagen, proteoglycans and elastin in atheroma, see Table

1. In atherosclerotic plaques three main types are found: metallo-proteinases (i.e. MMPs), serine proteases and cysteine proteases (i.e. cathepsins). Cathepsins and MMPs are responsible for degradation of all extracellular matrix proteins. As matrix is essential for plaque stability, its removal from the fibrous cap by proteases may invoke plaque rupture (Stary H. C.).

[0031] In Table 1 a variety of proteases found in atherosclerotic plaque are listed.

TABLE 1

Proteases detected in atherosclerotic plaques.

Protease	Degradation substrates
Cathepsin K	Proteoglycans, elastin, collagen
Cathepsin S	Proteoglycans, elastin, collagen
Cathepsin L	Proteoglycans, Collagen type I
Cathepsin B	Proteoglycans
MMP-1	Collagen type I, II and III
MMP-2	Proteoglycans, elastin
MMP-3	Proteoglycans, collagen type III, elastin
MMP-8	Proteoglycans, collagen type I, II and III
MMP-9	Elastin, collagen type I and III
MMP-13	Proteoglycans, collagen type I, II and III
MMP-18	Collagen type I

[0032] The main source of MMP expression in the plaque is suspected to be related to macrophage and SMC activity. Macrophages in plaques contain abundant MMP-1, -8, -9, and -13 and co-localize with sites of collagen and proteoglycan degradation in situ (Kunz J.). Furthermore, own data suggest localization of MMP-8 and Cathepsin K in atherosclerotic plaques.

Matrix Metalloproteinases (MMP)

[0033] MMP is a large group of endopeptidases, capable of degrading most components of the ECM. Presently, more than 25 MMPs have been identified. Metallo-proteinases are characterized by an active site containing a metal atom, typically zinc, and are secreted as zymogens. Specific tissue inhibitors, TIMPs, regulate the activity of MMPs. A great variety of MMPs are found in the atherosclerotic plaques. They are most often located in macrophages bordering the fibrous cap, within plaque shoulders in SMC and macrophages and are rarely identified within the fibrous cap (Kunz J.).

[0034] MMPs are classified in different groups according to their substrate specificity: Collagenases, which degrade fibrillar collagen, like collagen type I, II, III and V but also proteoglycans; Gelatinases, which degrade proteoglycans, collagen type IV, V, VII and elastin; Stromelysin that is active against proteoglycans and elastin (Rouis M). These three subgroups are of particular interest with regards to matrix remodelling in atherosclerotic plaques.

Gelatinases

[0035] Insoluble elastin is digested by MMP-2 and -9, both belonging to the gelatinase-family of MMPs. MMP-9 has an important role affecting the size and composition of atherosclerotic plaque. In unstable human atherosclerotic plaques and in vulnerable regions of plaques, greater expression and concentration of MMP-9 have been observed. Moreover, MMP-9 is found intracellularly (indicating active synthesis) in coronary plaques more often in patients with unstable angina compared with those with stable angina. Blood MMP-9 level increases in association with coronary atherosclerosis and predicts adverse cardiovascular events (Sundstrom and Vasan). A recent study by Kuzuya et al (2006) indicates that MMP-2 is responsible for accumulation of SMC in the fibrous cap and thereby inducing plaque instability.

Stromelysin

[0036] MMP-3 belongs to the stromelysin proteases and is capable of degrading both elastin and proteoglycans. A study by Yamada et al (2002) indicates that MMP-3 may prove to be a reliable mean of predicting the genetic risk of myocardial infarction in women. Elevations of stromelysin occur in osteoarthritis, rheumatoid arthritis, atherosclerotic lesions, gout, inflammatory bowel disease (IBD), idiopathic pulmonary fibrosis (IPF), certain cancers, joint injuries, and numerous inflammatory diseases.

Collagenases

[0037] MMP-1, -8 and -13 have all been identified in atherosclerotic plaques where they degrade proteoglycans and collagen types I and III.

[0038] MMP-1, -8 and -13 are collagenases, which cleave collagen into two fragments that are further degraded by MMP-2, -3 or -9.

[0039] MMP-8 is expressed by neutrophils, not commonly found in human atheroma but has been identified in atherosclerotic plaques. MMP-8 may be partly responsible for degradation of the fibrous cap as MMP-8 has a preference for collagen type I (Herman et al), having a three fold greater activity in degradation of collagen I than MMP-1 and 13. This is supported by Turu et al (2006), in this study the content of MMP-8 in the plasma are significantly higher for patients with vulnerable plaques, than patients with stable plaques.

[0040] MMP-13 has been reported to cleave SLRPS, with high specificity for biglycan. Degradation of biglycan by MMP-13 at a specific cleavage site (. . . G_{177}/V_{178}) has previously been demonstrated by Monfort et al. (2005) and proposed to play a important role in early detection of cartilage degradation in osteoarthritis.)

Cathepsins

[0041] Human cysteine cathepsins consist of 11 members, including cathepsins B, K, L, and S, and are predominantly expressed within the endosomal/lysosomal compartments of cells. Cathepsins are capable of catalysing the hydrolytic breakdown of proteoglycans, collagen and elastin.

[0042] In abdominal aortic aneurysm (AAA) high levels of cathepsins S, K, and L were found compared to normal aorta. Normal human vascular SMC contain no detectable cathepsin K by immunostaining, but cells within atherosclerotic plaques are clearly positive. Cathepsin K is localized in rupture-prone areas such as the fibrous cap, plaque shoulders and at the actual site of plaque ruptures (Chapman et al). Cathepsin S is found to co-localize with regions of increased elastin breakdown in atherosclerotic plaques, and reduced atherosclerosis is observed in cathepsin S- and K-deficient mice (Liu et al).

[0043] Both cathepsin L and K degrade several proteoglycans and collagen type I and II, cathepsin K degrades within covalently cross-linked triple helices, while cathepsin L

cleaves only in the nonhelical telopeptide regions. Cathepsin K is localized in the fibrous cap and plaque shoulder. Cathepsin K expression in normal arteries is very low. Early human atherosclerotic lesions showed cathepsin K expression in the intimal and medial SMCs. In advanced atherosclerotic plaques, cathepsin K was localized mainly in macrophages and SMCs of the fibrous cap (Lutgens et al). Cathepsin K protein levels were increased in atherosclerotic lesions when compared with normal arteries, whereas cathepsin K mRNA levels were similar in both atherosclerotic and normal arteries. Furthermore, it was shown that cathepsin K mRNA and protein levels were highest in advanced but stable human atherosclerotic plaques compared with early atherosclerotic lesions and lesions containing thrombus (Chapman et al).

[0044] Cathepsin S is only sparsely expressed in intimal and medial SMCs in early human atherosclerotic lesion and fatty streaks. In advanced human atherosclerotic plaques cathepsin S was localized in macrophages and SMCs of the fibrous cap. EC lining the lumen of the vessel itself and the plaque microvessels also expressed cathepsin S. Furthermore, cathepsin S mRNA and protein levels were increased in human atheroma compared with normal arteries (Lutgens et al). Cathepsin S can degrade proteoglycans, elastin and collagen (Liu et al).

[0045] Presently, the determination of CVD risk is occurring at a late stage in atherosclerosis progression; a point in which there is a significant risk of fibrous plaque rupture. There is a need for diagnostic or prognostic assays that will provide information regarding atherosclerosis or CVD risk at both earlier stage and late stages. The findings of Katsuda et al (1992) suggest that there are enzymatic mechanisms for removal of collagens from advanced lesions, suggesting indeed a major role of neo-epitopes in arteriosclerosis.

Assays for Detection of Aggrecan and its Fragments

[0046] Several immunoassays for detection of aggrecan and its fragments have been described, however, none of these assays have been demonstrated to be useful for assessment of CVD. Most such assays are concerned with cartilage degradation, but U.S. Pat. No. 5,387,504 describes the neo-epitope VDIPEN released by the action of stromelysin at the site N_{341}-F_{342} of aggrecan and an RIA assay employing a single monoclonal antibody specific for this epitope. It is alleged that such an assay might be useful in the diagnosis of various diseases including atherosclerotic lesions and also that aggrecan fragments arising from cartilage will be detectable this way in synovial fluid, blood, urine or other biological fluids as a measure of stomelysin activity or the effect of a stromelysin inhibitor. However, there is no data presented to support these suggestions. Nor are we aware of any being subsequently published.

[0047] RIA immunoassays for the VIDIPEN sequence have been available for many years now and have never been shown to be useful for measuring VIDIPEN containing peptides in blood or other similar body fluids as a diagnostic for any indication.

[0048] U.S. Pat. No. 5,387,504 indicates that the N-terminal sequence generated by cleavage at the N_{341}-F_{342} site may remain attached to cartilage, or may be released into synovial fluid. It suggests that assays to measure such fragments may be useful in characterising stomolysin inhibitors by monitoring release of such fragments into fluids including blood. The production of rabbit polyclonal antiserum useful to quantify large fragments from cartilage bearing the N-terminal sequence FFVG . . . is disclosed. General protocols for obtaining monoclonal antibodies are described also.

[0049] Numerous proteolytic clevage-sites have been described for aggrecan (Fosang et al ., 2000; Caterson et al., 2000), and a predominant site for the metalloproteinases (MMPs) is located in the intra-globular domain (IGD) between amino acid N^{341} and F^{342} (Fosang et al. 1996). A monoclonal antibody, i.e. AF28 (ATCC HB11671), that specifically binds the polypeptide neo-epitope containing the N-terminal sequence ^{342}FFGVG . . . , has previously been developed (Fosang et al. 1995). The AF28 antibody has been used in competition ELISA for detection of aggrecan fragments in synovial fluid and human serum (Fosang et al. 1995).

[0050] Sumer et al. (2006) discloses two immunoassays, one of them detecting aggrecan fragments carrying both the neo-epitope ^{342}FFGVG and the globular domain G2. A capture antibody (AF28) binding to the neo-epitope ^{342}FFGVG was biotinylated and incubated on streptavidin-coated microtitre plates. Another antibody (F78) binding the G2 domain of aggrecan (but also having the ability to bind the G1 domain) was labelled with horseradish peroxidase, and used as detector antibody. Levels of the ^{342}FFGVG-G2 fragments are slightly elevated in patients with rheumatoid arthritis, however, it did not reach statistical significance above a control population. Measurement of aggrecan fragments in patients with CVD symptoms was not reported.

[0051] Pratta et al. (2006) discloses a sandwich assay for detection of aggrecan fragments employing a monoclonal antibody to keratan sulfate as capture antibody and another monoclonal antibody to the neo-epitope ARGSVIL as detector antibody. This assay detects aggrecan fragments in human synovial fluid, however, measurements in human serum or plasma have not been reported and relevance to CVD is not suggested.

[0052] Karsdal et al. (2008) describes an immunoassay similar to that of Sumer above, except that the capture antibody was substituted with a monoclonal antibody (BC-3) binding to the neo-epitope ^{374}ARGSVIL. The test was not used for measurements in human serum and no reference was made to relevance to CVD diagnosis.

[0053] More generally the use of monospecific antibodies specific for fragments of aggrecan, generated by specific stromelysin cleavage have been described. Until now, the clinical value of assays specific for these aggrecan 'neo-epitopes' has not been established and neither has it previously been established whether these fragments are released into circulation in significant amounts and how they are catabolised.

[0054] The CS 846 test uses antibodies recognising the chondroitin sulfate sidechain bound to amino acid 846 between the G2 and the G3 domain of the aggrecan molecule (IBEX Pharmaceuticals Inc.) (Glant et al., 1986; Rizkalla et al., 1992; Månsson et al., 1995). It is reported to be an assay for elvated fetal like aggrecan synthesis. The FA-846 sandwich immunoassay, which is an adaptation of the CS 846 test for the quantification of fetal aggrecan, has also been described.

[0055] Other tests for aggrecan have been developed, e.g. "Aggrecan Proteoglycan" (Biosource, US). However, the specificity of the antibodies remains to be determined. Other aggrecan assays target the glycosaminoglycan region of aggrecan, i.e. between the G2 and the G3 domain (Kongtawelert and Ghosh 1990).

[0056] Møller et al. have developed a competition ELISA for the core protein part of aggrecan, though not specifying the binding region of the antibody (Møller et al. 1994).

[0057] The antibody, l-C-6 has been developed which binds to both the G1 and un-masked G2 domains (Fosang and Hardingham, 1991). The G2 domain masking keratan sulphate side chains had to be removed using keratanase for reactivity with l-C-6 with the G2 domain. Accordingly, the l-C-6 antibody is not suitable for use in assays for aggrecan or aggrecan fragments in body fluids or body tissues.

[0058] Aggrecan is referred to in a number of patent publications. Several of these refer to measurement of aggrecan or certain characteristic fragments of the protein with a diagnostic purpose to assess cartilage catabolism.

[0059] U.S. Pat. No. 4,704,356 discloses that abnormal levels of keratan sulfate (KS) in the peripheral blood are indicative of abnormalities of cartilage or cartilage-like tissues. Elevated levels of KS in the peripheral blood are described as being indicative of osteoarthritis. Interestingly absence of KS as well as very elevated levels of KS in the peripheral blood were found to be indicative of muscular dystrophy and related disorders. The technique used for quantification of KS in the peripheral blood was an immunoassay using a monoclonal antibody.

[0060] U.S. Pat. No. 5,935,796 describes other diagnostic methods and compositions relating to the proteoglycan proteins of cartilage breakdown. Methods are described for early diagnosis, monitoring and treatment of osteoarthritis using monoclonal antibodies which specifically recognize antigenic determinants on atypical chondroitin sulfate (CS)/dermatan sulfate glycosaminoglycan chains in body tissues and fluids, that originate from articular cartilage aggrecan.

[0061] U.S. Pat. No. 4,778,768 describes methods for monitoring the progressive destruction of articular cartilage in joints, and more specifically for determining changes occurring in articular cartilage. The method involves (a) quantifying proteoglycan monomer and/or antigenic fragments thereof in a synovial fluid sample and (b) correlating the values thus obtained with progressive destructions in the articular cartilage appertaining to that sample fluid. The proteoglycan fragments were measured by an immunoassay employing an antibody specific to proteoglycan monomers. The assay described in this patent appears to be identical with the polyclonal HABr ELISA described above.

[0062] U.S. Pat. No. 5,948,692 describes an assay, which uses a size separation method for dividing glycans having avidity for hyaluronic acid (HA) from proteoglycans not having such avidity. The assay measures the HA binding proteoglycans, such as aggrecan. This is said to enable the biochemical diagnosis of joint diseases in the field of orthopedics as well as RA, OA and other joint diseases. The method can it is said be utilized also for discriminating normal joints from pathologic joints, for providing a prognostic measure of disease progression and for monitoring the effects of therapeutic interventions.

[0063] U.S. Pat. No. 5,427,954 describes the use of an immunoassay for measurement of aggrecan containing a neo-epitope ARGSVI. This is one of a number of disclosures describing the diagnostic utility of neo-epitopes generated by specific proteolysis of aggrecan mediated by proteases involved in the pathological processes of joint diseases.

[0064] U.S. Pat. No. 5,935,796 relates to methods and compositions for early diagnosis, monitoring and treatment of cartilage degenerative conditions, using an antibody which recognizes a peptide comprising the sequence FFGVG generated by cleavage of cartilage aggrecan at the site N_{341}-F_{342}. This epitope is the 'other end' of the VDIPEN epitope released by the action of stromelysin on aggrecan. It is suggested to provide a sandwich assay to improve the sensitivity of detection of FFGVG fragments of aggrecan, more specifically a sandwich assay combining AF-28 with an anti-keratan sulphate antibody such as 5-D-4.

[0065] U.S. Pat. No. 5,185,245 describes an immunoassay for detection of proteoglycans in synovial fluid and methods of monitoring treatment of diseases characterised by breakdown of proteoglycans. A test sample of synovial fluid is quantified by an immunoassay employing antibodies specifically recognizing proteoglycan, where the antibodies are immobilized on a solid support. Bound proteoglycan is then contacted with a second specific antibody, which is labeled with a detection reagent (i.e. peroxidase). Both antibodies have affinity to the glycosaminoglycan (GAG/CS) moieties on the proteoglycan.

[0066] U.S. Pat. No. 5,354,662 and U.S. Pat. No. 5,217,903 describe generally the measurement of 'tissue breakdown products' in body fluids based on quantification of a connective tissue or muscle tissue breakdown product in a body fluid from an animal by using a standard comprising the breakdown product having a radioactive label. The standard should have a known specific activity and thus combining the standard and a sample of the body fluid, the specific radioactivity measured in a RIA/IRMA type assay can be used as a measure of the quantity of the breakdown product in the sample. Also described are methods for assessing, in a body fluid from an animal, the condition of a selected connective tissue or a muscle tissue in an animal, and for assessing a disease process that includes destruction of a specified connective tissue component or muscle tissue, and for assessing the efficacy of a therapy for treatment of such a disease process, include the steps of the method for determining the quantity of a tissue breakdown product.

[0067] WO2007/045661 discloses assays for detection of aggrecan fragments using antibodies recognising epitopes located on the G2 domain of aggrecan, which are able to bind to aggrecan without prior removal of keratan sulfate by keratanase and using neo-epitope recognising antibodies. The patent application does not disclose the use of detecting aggrecan fragments for assessment of CVD.

BRIEF SUMMARY OF THE INVENTION

[0068] It has now surprisingly been discovered that the concentration of certain aggrecan fragments is markedly elevated in certain clinical conditions associated with CVD, including coronary heart disease.

[0069] The present invention provides a method of diagnosis of cardiovascular disease (CVD) comprising a method of diagnosis of cardiovascular disease (CVD) comprising obtaining a patient biofluid sample, conducting an immunoassay to measure aggrecan fragments in said sample, and associating an elevation of said measure in said patient above a normal level with the presence of CVD, wherein said immunoassay is conducted by a method comprising: contacting aggrecan fragments in said sample with an first immunological binding partner reactive with an N-terminal first epitope formed by cleavage of aggrecan by a proteinase and with a second immunological binding partner reactive with a second aggrecan epitope which is present in aggrecan at a location in the C-terminal direction from the location of said N-termainal epitope, and measuring the extent of simultaneous binding of aggrecan fragments to both said first and said second immu-

nological binding partners to measure therein aggrecan fragments comprising both of said first and said second epitopes.

[0070] The result of said assay may produce an index indicative of the degree of risk in a particular patient of rupture of an atherosclerotic plaque or of the vulnerable status of the atherosclerotic plaques of a patient.

[0071] Patients having a value for said index above a threshold level may be recommended for further investigation by plaque imaging methods (including those discussed above) or for the prescribing of medication for treatment of atherosclerosis or for surgical treatment of atherosclerosis, and such follow up investigations or treatment may form part of the method of the invention. Alternatively they may be selected for inclusion in a clinical trial of a therapeutic entity.

[0072] The method according to the invention may include comparing the measured amount of aggrecan fragments with a previously measured range of comparable values obtained for samples from a first group of patients having no cardiovascular disease and from a second group of patients previously diagnosed as having cardiovascular disease.

[0073] The method of the invention may include conducting the defined assay on samples derived from a first comparator patient group having no cardiovascular disease and conducting the defined assay on samples derived form a second comparator patient group having known cardiovascular disease, to obtain said range of comparable values.

[0074] The neoepitope is preferably located between the G1 and G2 domains.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Aggrecan Assays

[0075] Aggrecan fragments may be fragments generated by any of the proteases present in atherosclerotic arteries.

[0076] Several candidate proteases may be responsible for the digestion of aggrecan in the plaque, as the literature reports many different proteases in the atherosclerotic plaques. Most likely, this is the result of a large range of complicated processes eventually leading to plaque rupture. However, in our assessment, early phases may consist of a range of MMPs, whereas later stages may rely more on cathepsin degradation of the matrix, resulting in different neoepitope profiles dependent on the stages of the disease. Also, aggrecanases are present in atherosclerotic plaques and could therefore generate aggrecan fragments.

List of protease sites in aggrecan (AG)

TABLE 2

Protease sites in the interglobular domain of aggrecan.

AG sequence	SEQ ID NO	Cleavage Enzyme
VDIPEN*FFGVGG	2	
NITEGE*ARGSVI	3	
ILTVKP*IFEVSP	4	
AFTSED*LVVQVT	5	
AFCFRG*ISAVPS	6	

*indicates a site ofcleavage.

[0077] Accordingly, in a method of the invention, said aggrecan fragments preferably comprise an N-terminal neoepitope formed by cleavage of aggrecan by a protease at a site marked by the sign * in any one of the above partial sequences thereof.

[0078] Preferably, said first immunological binding partner is not reactive with other PGs. Preferably, said immunological binding partner is not reactive with a said sequence listed above if prolonged past the respective N-terminal ends of generated fragments.

[0079] Suitable immunological binding partners may therefore be specifically reactive with any of the following sequences in Table 3 at the N terminal of a peptide:

TABLE 3

N-terminal sequences of protease generated peptide fragments of aggrecan

Aggrecan neo-epitope, N-term.	SEQ ID NO
*FFGVGGEEDI	7
*ARGSVILTVK	8
*IFEVSPSPLE	9
*LVVQVTAVPG	10
*ISAVPSPGEE	11

[0080] Further cleavage sites defining neo-epitopes that may be assayed in a similar manner can be identified by exposing aggrecan to any of the enzymes described herein and isolating and sequencing peptides thereby produced.

[0081] In particular, aggrecan fragments carrying both one of the above mentioned neo-epitopes and a larger globular domain, e.g. globular domain 2, have been demonstrated to be particular useful for detecting abnormalities associated with CVD.

[0082] Therefore, in one embodiment of the invention, the assay uses two antibodies (or more generally, immunological binding partners) in a sandwich construction, one antibody detecting the neo-epitope and the other antibody binding to the globular domain of aggrecan.

[0083] Aggrecan specificity is not necessary however in the case of the second antibody, because it is adequately provided by the first antibody. Accordingly, the second antibody may be one which is specifically reactive with the aggrecan fragments containing the first epitope, but by virtue of being reactive with a structure which is not unique to aggrecan, for instance by binding keratan sulphate.

[0084] Assays for more than one of the peptides described above may be conducted separately and their results combined or more than one of the peptides described above may be measured together.

[0085] The result of an assay according to the invention may be combined with one or more other measured biomarkers to form a composite index of diagnostic or prognostic value.

[0086] The term 'immunological binding partner' as used herein includes polyclonal and monoclonal antibodies and also specific binding fragments of antibodies such as Fab or $F(ab')_2$. Thus, said immunological binding partner may be a monoclonal antibody or a fragment of a monoclonal antibody having specific binding affinity.

[0087] Generally, all previously known sandwich immunoassay formats can be used in accordance with this invention including heterogeneous and homogeneous formats enzyme linked assays, radio-immune assays and the like.

[0088] A suitable method could be a sandwich assay using two different antibodies, preferable monoclonal antibodies. One antibody binding to the neo-epitope as described above, and the other antibody recognising one of the globular domains of aggrecan, i.e. globular domain 2 (G2) or 3 (G3). The neo-epitope binding antibody could be used for coating of microtitre plates, which are subsequently incubated with a sample suspected to contain aggrecan fragments derived from atherosclerotic plaques. Next, the wells of the plates are incubated with the second antibody binding to G2 or G3, and this antibody could be labelled for detection, e.g. with horseradish peroxidise or other suitable label.

[0089] In certain preferred methods, the sample is a patient derived sample, and the method further comprises comparing the determined level of said binding of said peptide fragments with values characteristic of (a) comparable healthy individuals and/or (b) a pathological atherosclerotic condition and optionally associating a higher level of the measured peptide (normally indicated by a higher level of binding) with a more severe degree of a said condition.

[0090] An aspect of the present invention relates to the development of monoclonal antibodies recognising neo-epitopes as described above. This can be achieved by immunising mice with synthetic peptides originating from the amino acid sequence of the protein molecule concerned (including the sequences listed above or sequences terminating therein), fusing the spleen-cells from selected mice to myeloma cells, and testing the monoclonal antibodies for binding to neo-epitopes on relevant synthetic peptides. Specificity for neo-epitopes can be ensured by requiring reactivity with a synthetic peptide and a lack of reactivity with either a C-prolongated form of the immunising peptide (for a C-terminal neo-epitope) or an N-terminal prolongated form of the immunising peptide (for an N-terminal neo-epitope). Antibodies for neo-epitopes may also be evaluated to establish a lack of binding capacity to native protein. Alternatively, specificity for a neo-epitope can be ensured by requiring the reactivity of the antibody to be negatively dependent on the presence of biotin or other functional groups covalently linked to one of the terminal amino acids.

[0091] The invention will make use of an immunological binding partner which is specifically immunoreactive with a neo-epitope formed by cleavage of aggrecan by a protease at an end-site in any one of the partial sequences set out above, and may be for instance a monoclonal antibody or a binding fragment thereof.

[0092] The invention may make use of a cell line producing a monoclonal antibody against an N-terminal neo-epitope formed by cleavage of an atherosclerotic plaque protein at the end-sites of sequences in any one of the partial sequences o set out above.

[0093] The invention may further make use of a peptide comprising an N-terminal neo-epitope formed by cleavage of aggrecan in any one of the partial sequences of these proteins set out above. Such a peptide may be conjugated as a hapten to a carrier for producing an immune response to said peptide, or immobilised to a solid surface or conjugated to a detectable marker for use in an immunoassay.

[0094] The invention may employ methods for the development of monoclonal antibodies recognising the globular domains of aggrecan as described above. This can be achieved by immunising mice with purified, intact aggrecan, fusing the spleen-cells from selected mice to myeloma cells, and testing the monoclonal antibodies for reactivity to intact aggrecan. Specificity for aggrecan can be ensured by demonstrating lack of reactivity to other proteoglycans, e.g. by showing that the binding of the monoclonal antibodies to aggrecan cannot be inhibited by coincubation with said other proteoglycans.

[0095] The invention may make use of an immunological binding partner which is specifically immunoreactive with one of the globular domains, i.e. G2 or G3, and may be for instance a monoclonal antibody or a binding fragment thereof.

[0096] The invention may make use of a cell line producing a monoclonal antibody against intact aggrecan.

[0097] The invention may further make use of an isolated nucleic acid molecule coding for a peptide comprising an N-terminal neo-epitope formed by cleavage of aggrecan in any one of the partial sequences set out above.

[0098] The invention may further make use of a vector comprising a nucleic acid sequence comprising an expression signal and a coding sequence which codes for the expression of a peptide comprising an N-terminal neo-epitope formed by cleavage of aggrecan in any one of the partial sequences set out above and further includes a host cell transformed with such a vector and expressing a said peptide.

[0099] The invention may be performed using kits, which may include (1) a microtitre plate coated with synthetic peptide; (2) a monoclonal antibody or antibody binding fragment of the invention reactive with said synthetic peptide characteristic of aggrecan; and (3) a labelled anti-mouse IgG immunoglobulin. Alternatively, such kits may include (1) a microtitre plate coated with purified native aggrecan fragments; (2) a monoclonal antibody recognising a neo-epitope on an aggrecan fragment, and reactive with said purified fragments; and (3) a labelled anti-mouse IgG immunoglobulin. Alternatively, such kits may include (1) a microtitre plate coated with streptavidin; (2) a synthetic peptide linked to biotin; (3) a monoclonal antibody recognising a neo-epitope on said aggrecan fragment and reactive with said synthetic peptide; and (4) a labelled anti-mouse IgG immunoglobulin. Yet another alternative could be kits including (1) a microtitre plate coated with streptavidin; (2) a synthetic peptide linked to biotin; (3) a monoclonal antibody recognising a neo-epitope on said aggrecan fragment (and reactive with said synthetic peptide) and conjugated to horseradish peroxidase. And yet another alternative, such kits may include; (1) a microtitre plate coated with a monoclonal antibody binding to a neo-epitope on aggrecan; (2) a monoclonal antibody recognising G2 or G3 on said aggrecan fragment and conjugated to horseradish peroxidise. Another alternative include kits containing; (1) a microtitre plate coated with streptavidin; (2) a biotin-labelled monoclonal antibody binding to a neo-epitope on said aggrecan fragment (3) a monoclonal antibody recognising G2 or G3 on said aggrecan fragment.

[0100] Thus, the invention may make use of an immunoassay kit comprising an immunological binding partner as described herein, and a competition agent which binds said immunological binding partner, and optionally one or more of a wash reagent, a buffer, a stopping reagent, an enzyme label, an enzyme label substrate, calibration standards, an anti-mouse antibody and instructions for conducting a said immunoassay.

[0101] Also, the invention may employ an immunoassay kit comprising two different immunological binding partners as described herein, and optionally one or more of a wash reagent, a buffer, a stopping reagent, an enzyme label substrate, and calibration standards, and instructions for conducting a said immunoassay.

[0102] The assays described herein are useful in the diagnosis of atherosclerotic disease in patients. In addition, the tests are useful for the assessment of disease progression, and the monitoring of response to therapy. The immunological binding partners of the invention may also be used in immunostaining to show the presence or location of cleavage products of any atherosclerotic plaque protein described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[0103] The invention will be further explained and illustrated with reference to the accompanying drawings, in which:

[0104] FIG. **1** shows the structure of aggrecan.

[0105] FIGS. **2**A, B, C and D show the detection of aggrecan fragments in serum samples from patients with congestive heart failure (CF) and coronary heart disease (CHD) using two assays according to the invention and two comparative methods. Control samples include serum samples from patients with congestive heart failure.

EXAMPLE 1

^{342}FFGVG-G2 Assay

[0106] An immune assay for detection of aggrecan fragments carrying both the neo-epitope ^{342}FFGVG and the globular domain G2 was conducted as described by Sumer et al., 2006. Briefly, monoclonal antibody Af28 binding to the neo-epitope ^{342}FFGVG (U.S. Pat. No. 5,935,796) was labelled with biotin and used for coating of streptavidin plates After incubating the plates for 1 hour, plates were washed 5 times with washing buffer (0.15 mol/1 NaCl, 0.05% (v/v) Tween 20). Subsequently 50 µl standards (MMP-13 digested purified bovine aggrecan (SIGMA) 47-3000 ng/ml or human serum prediluted 1:50 in PBS-BTB were added, and the plates were incubated for 1 hour, 300 RPM, 20° C. After the incubation period, the plates were washed 5 times as described previously, and 500 ng/ml horseradish Peroxidase (POD)-labelled F78 antibody diluted in PBS-BTB with blocking agent (Roche GmbH) was added. After incubating for 1 hour, 300 RPM, 20° C., the plates were washed 5 times, 100 µl of TMB substrate was added, and the plates were incubated for 15 minutes, 300 RPM, 20° C. in the dark before 150 µl 0.18 M H_2SO_4 was added. The absorbance was measured immediately after at 450 nm.

[0107] Human serum samples from patients with congestive heart failure (CF) and coronary heart disease (CHD) was measured in the assay. Control specimens originated from patients with various inflammatory diseases, including rheumatoid arthritis. Also, as control, the serum samples was evaluated for presence of aggrecan fragments containing G1 and/or G2 using antibody F78 as both capture and detector antibody.

[0108] The test was carried out on two groups of samples, including in each group samples from patients with known CVD and patients with known CHD. In FIGS. **2**A-D, results for the two groups are shown. A dramatic elevation in the serum concentration of 342FFGVG-G2 fragments was detected in patients with CHD (FIG. **2**A). In contrast, only background levels of these fragments were detected in patients with CF and the control population.

EXAMPLE 2

374ARGSV-G2 Sandwich ELISA

Aggrecanase-Derived Aggrecan Fragments Carrying the 374ARGSV Neo-Epitope and the G2 Domain

[0109] Microtitre plates were coated with rabbit anti-mouse immunoglobulins diluted to 10 µg/ml and incubated overnight at 4° C., After washing, the wells were incubated with monoclonal antibody 6D6 diluted in to 500 ng/ml in PBS-BTE (PBS with 1% (w/v) BSA Tween 20 and EDTA, pH 7.4). MAb 6D6 binds specifically to the N-terminal neo-epitope ^{374}ARGSV generated by proteolytic cleavage of aggrecan by aggrecanase. Following incubation for 1 hour at 20° C. with shaking and washing, the wells were incubated for another hour with 100 µL of human serum pre-diluted 1:20 in PBS-BT2 buffer (as PBS-BT1 but with 8 g/L of NaCl). Bovine aggrecan cleaved for 24 hours with ADAMTS-4 and diluted in PBS-BT2 buffer was used as calibrators. Bound antigen was detected by incubation with POD-labelled MAb F78 diluted to 2000 ng/ml in PBS-BT1 buffer (PBS with BSA, 0.8 g/L NaCl, and Tween 20) containing 10% Liquid II (Roche GmbH) for 1 hour at 20° C. with shaking. After washing the colour reaction was performed as described above.

[0110] Serum samples also used in Example 1 were evaluated in the 374ARGSV-G2 sandwich ELISA. Patients with coronary heart disease (CHD) had a concentration of circulating aggrecan fragments of 4330+194 ng/ml, whereas samples from patients with congestive heart failure (CHF) had 3020+372 ng/ml ($p<0.0001$), as shown in FIG. **2**C.

COMPARATIVE EXAMPLES

[0111] By way of comparison, ELISAs were conducted on the same serum samples to measure fragments comprising the G1 globular domain of aggrecan and a C-terminal neo-epitope which was . . . VIDIPEN in one case and . . . NITEGE in another. The results are seen in FIGS. **2**B and C. No statistically significant difference is found in comparing CVD and CHF patients.

[0112] In this specification, unless expressly otherwise indicated, the word 'or' is used in the sense of an operator that returns a true value when either or both of the stated conditions is met, as opposed to the operator 'exclusive or' which requires that only one of the conditions is met. The word 'comprising' is used in the sense of 'including' rather than in to mean 'consisting of'. All prior teachings acknowledged above are hereby incorporated by reference. No acknowledgement of any prior published document herein should be taken to be an admission or representation that the teaching thereof was common general knowledge in Australia or elsewhere at the date hereof.

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[0146] The entire contents of all patents, published patent applications and other references cited herein are hereby expressly incorporated herein in their entireties by reference.

[0147] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures described herein. Such equivalents are considered to be within the scope of this invention and are covered by the following claims.

SEQUENCES REFERRED TO ABOVE:

SEQ ID NO 1

```
   1 MTTLLWVFVT LRVITAAVTV ETSDHDNSLS VSIPQPSPLR VLLGTSLTIP CYFIDPMHPV

  61 TTAPSTAPLA PRIKWSRVSK EKEVVLLVAT EGRVRVNSAY QDKVSLPNYP AIPSDATLEV

 121 QSLRSNDSGV YRCEVMHGIE DSEATLEVVV KGIVFHYRAI STRYTLDFDR AQRACLQNSA

 181 IIATPEQLQA AYEDGFHQCD AGWLADQTVR YPIHTPREGC YGDKDEFPGV RTYGIRDTNE

 241 TYDVYCFAEE MEGEVFYATS PEKFTFQEAA NECRRLGARL ATTGHVYLAW QAGMDMCSAG

 301 WLADRSVRYP ISKARPNCGG NLLGVRTVYV HANQTGYPDP SSRYDAICYT GEDFVDIPEN

 361 FFGVGGEEDI TVQTVTWPDM ELPLPRNITE GEARGSVILT VKPIFEVSPS PLEPEEPFTF

 421 APEIGATAFA EVENETGEAT RPWGFPTPGL GPATAFTSED LVVQVTAVPG QPHLPGGVVF

 481 HYRPGPTRYS LTFEEAQQAC PGTGAVIASP EQLQAAYEAG YEQCDAGWLR DQTVRYPIVS

 541 PRTPCVGDKD SSPGVRTYGV RPSTETYDVY CFVDRLEGEV FFATRLEQFT FQEALEFCES

 601 HNATATTGQL YAAWSRGLDK CYAGWLADGS LRYPIVTPRP ACGGDKPGVR TVYLYPNQTG

 661 LPDPLSRHHA FCFRGISAVP SPGEEEGGTP TSPSGVEEWI VTQVVPGVAA VPVEEETTAV

 721 PSGETTAILE FTTEPENQTE WEPAYTPVGT SPLPGILPTW PPTGAETEES TEGPSATEVP

 781 SASEEPSPSE VPFPSEEPSP SEEPFPSVRP FPSVELFPSE EPFPSKEPSP SEEPSASEEP

 841 YTPSPPEPSW TELPSSGEES GAPDVSGDFT GSGDVSGHLD FSGQLSGDRA SGLPSGDLDS

 901 SGLTSTVGSG LTVESGLPSG DEERIEWPST PTVGELPSGA EILEGSASGV GDLSGLPSGE

 961 VLETSASGVG DLSGLPSGEV LETTAPGVED ISGLPSGEVL ETTAPGVEDI SGLPSGEVLE

1021 TTAPGVEDIS GLPSGEVLET TAPGVEDISG LPSGEVLETT APGVEDISGL PSGEVLETAA

1081 PGVEDISGLP SGEVLETAAP GVEDISGLPS GEVLETAAPG VEDISGLPSG EVLETAAPGV

1141 EDISGLPSGE VLETAAPGVE DISGLPSGEV LETAAPGVED ISGLPSGEVL ETAAPGVEDI

1201 SGLPSGEVLE TAAPGVEDIS GLPSGEVLET AAPGVEDISG LPSGEVLETA APGVEDISGL

1261 PSGEVLETAA PGVEDISGLP SGEVLETTAP GVEEISGLPS GEVLETTAPG VDEISGLPSG

1321 EVLETTAPGV EEISGLPSGE VLETSTSAVG DLSGLPSGGE VLEISVSGVE DISGLPSGEV

1381 VETSASGIED VSELPSGEGL ETSASGVEDL SRLPSGEEVL EISASGFGDL SGVPSGGEGL

1441 ETSASEVGTD LSGLPSGREG LETSASGAED LSGLPSGKED LVGSASGDLD LGKLPSGTLG

1501 SGQAPETSGL PSGFSGEYSG VDLGSGPPSG LPDFSGLPSG FPTVSLVDST LVEVVTASTA

1561 SELEGRGTIG ISGAGEISGL PSSELDISGR ASGLPSGTEL SGQASGSPDV SGEIPGLFGV

1621 SGQPSGFPDT SGETSGVTEL SGLSSGQPGV SGEASGVLYG TSQPFGITDL SGETSGVPDL

1681 SGQPSGLPGF SGATSGVPDL VSGTTSGSGE SSGITFVDTS LVEVAPTTFK EEEGLGSVEL

1741 SGLPSGEADL SGKSGMVDVS GQFSGTVDSS GFTSQTPEFS GLPSGIAEVS GESSRAEIGS

1801 SLPSGAYYGS GTPSSFPTVS LVDRTLVESV TQAPTAQEAG EGPSGILELS GAHSGAPDMS

1861 GEHSGFLDLS GLQSGLIEPS GEPPGTPYFS GDFASTTNVS GESSVAMGTS GEASGLPEVT
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1921 LITSEFVEGV TEPTISQELG QRPPVTHTPQ LFESSGKVST AGDISGATPV LPGSGVEVSS

1981 VPESSSETSA YPEAGFGASA APEASREDSG SPDLSETTSA FHEANLERSS GLGVSGSTLT

2041 FQEGEASAAP EVSGESTTTS DVGTEAPGLP SATPTASGDR TEISGDLSGH TSQLGVVIST

2101 SIPESEWTQQ TQRPAETHLE IESSSLLYSG EETHTVETAT SPTDASIPAS PEWKRESEST

2161 AADQEVCEEG WNKYQGHCYR HFPDRETWVD AERRCREQQS HLSSIVTPEE QEFVNNNAQD

2221 YQWIGLNDRT IEGDFRWSDG HPMQFENWRP NQPDNFFAAG EDCVVMIWHE KGEWNDVPCN

2281 YHLPFTCKKG TATTYKRRLQ KRSSRHPRRS RPSTAH

SEQ ID NO 2
VDIPENFFGVGG

SEQ ID NO 3
NITEGEARGSVI

SEQ ID NO 4
ILTVKPIFEVSP

SEQ ID NO 5
AFTSEDLVVQVT

SEQ ID NO 6
AFCFRGISAVPS

SEQ ID NO 7
FFGVGGEEDI

SEQ ID NO 8
ARGSVILTVK

SEQ ID NO 9
IFEVSPSPLE

SEQ ID NO 10
LVVQVTAVPG

SEQ ID NO 11
ISAVPSPGEE

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 18

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<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

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1 5 10 15

Ala Val Thr Val Glu Thr Ser Asp His Asp Asn Ser Leu Ser Val Ser
20 25 30

Ile Pro Gln Pro Ser Pro Leu Arg Val Leu Leu Gly Thr Ser Leu Thr
35 40 45

Ile Pro Cys Tyr Phe Ile Asp Pro Met His Pro Val Thr Thr Ala Pro
50 55 60

Ser Thr Ala Pro Leu Ala Pro Arg Ile Lys Trp Ser Arg Val Ser Lys
65 70 75 80

Glu Lys Glu Val Val Leu Leu Val Ala Thr Glu Gly Arg Val Arg Val
85 90 95

-continued

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Asn Ser Ala Tyr Gln Asp Lys Val Ser Leu Pro Asn Tyr Pro Ala Ile
            100                 105                 110

Pro Ser Asp Ala Thr Leu Glu Val Gln Ser Leu Arg Ser Asn Asp Ser
        115                 120                 125

Gly Val Tyr Arg Cys Glu Val Met His Gly Ile Glu Asp Ser Glu Ala
    130                 135                 140

Thr Leu Glu Val Val Val Lys Gly Ile Val Phe His Tyr Arg Ala Ile
145                 150                 155                 160

Ser Thr Arg Tyr Thr Leu Asp Phe Asp Arg Ala Gln Arg Ala Cys Leu
                165                 170                 175

Gln Asn Ser Ala Ile Ile Ala Thr Pro Glu Gln Leu Gln Ala Ala Tyr
            180                 185                 190

Glu Asp Gly Phe His Gln Cys Asp Ala Gly Trp Leu Ala Asp Gln Thr
        195                 200                 205

Val Arg Tyr Pro Ile His Thr Pro Arg Glu Gly Cys Tyr Gly Asp Lys
    210                 215                 220

Asp Glu Phe Pro Gly Val Arg Thr Tyr Gly Ile Arg Asp Thr Asn Glu
225                 230                 235                 240

Thr Tyr Asp Val Tyr Cys Phe Ala Glu Glu Met Glu Gly Glu Val Phe
                245                 250                 255

Tyr Ala Thr Ser Pro Glu Lys Phe Thr Phe Gln Glu Ala Ala Asn Glu
            260                 265                 270

Cys Arg Arg Leu Gly Ala Arg Leu Ala Thr Thr Gly His Val Tyr Leu
        275                 280                 285

Ala Trp Gln Ala Gly Met Asp Met Cys Ser Ala Gly Trp Leu Ala Asp
    290                 295                 300

Arg Ser Val Arg Tyr Pro Ile Ser Lys Ala Arg Pro Asn Cys Gly Gly
305                 310                 315                 320

Asn Leu Leu Gly Val Arg Thr Val Tyr Val His Ala Asn Gln Thr Gly
                325                 330                 335

Tyr Pro Asp Pro Ser Ser Arg Tyr Asp Ala Ile Cys Tyr Thr Gly Glu
            340                 345                 350

Asp Phe Val Asp Ile Pro Glu Asn Phe Phe Gly Val Gly Gly Glu Glu
        355                 360                 365

Asp Ile Thr Val Gln Thr Val Thr Trp Pro Asp Met Glu Leu Pro Leu
    370                 375                 380

Pro Arg Asn Ile Thr Glu Gly Glu Ala Arg Gly Ser Val Ile Leu Thr
385                 390                 395                 400

Val Lys Pro Ile Phe Glu Val Ser Pro Ser Pro Leu Glu Pro Glu Glu
                405                 410                 415

Pro Phe Thr Phe Ala Pro Glu Ile Gly Ala Thr Ala Phe Ala Glu Val
            420                 425                 430

Glu Asn Glu Thr Gly Glu Ala Thr Arg Pro Trp Gly Phe Pro Thr Pro
        435                 440                 445

Gly Leu Gly Pro Ala Thr Ala Phe Thr Ser Glu Asp Leu Val Val Gln
    450                 455                 460

Val Thr Ala Val Pro Gly Gln Pro His Leu Pro Gly Gly Val Val Phe
465                 470                 475                 480

His Tyr Arg Pro Gly Pro Thr Arg Tyr Ser Leu Thr Phe Glu Glu Ala
                485                 490                 495
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Gln Gln Ala Cys Pro Gly Thr Gly Ala Val Ile Ala Ser Pro Glu Gln
            500                 505                 510

Leu Gln Ala Ala Tyr Glu Ala Gly Tyr Glu Gln Cys Asp Ala Gly Trp
        515                 520                 525

Leu Arg Asp Gln Thr Val Arg Tyr Pro Ile Val Ser Pro Arg Thr Pro
    530                 535                 540

Cys Val Gly Asp Lys Asp Ser Ser Pro Gly Val Arg Thr Tyr Gly Val
545                 550                 555                 560

Arg Pro Ser Thr Glu Thr Tyr Asp Val Tyr Cys Phe Val Asp Arg Leu
                565                 570                 575

Glu Gly Glu Val Phe Phe Ala Thr Arg Leu Glu Gln Phe Thr Phe Gln
            580                 585                 590

Glu Ala Leu Glu Phe Cys Glu Ser His Asn Ala Thr Ala Thr Thr Gly
        595                 600                 605

Gln Leu Tyr Ala Ala Trp Ser Arg Gly Leu Asp Lys Cys Tyr Ala Gly
    610                 615                 620

Trp Leu Ala Asp Gly Ser Leu Arg Tyr Pro Ile Val Thr Pro Arg Pro
625                 630                 635                 640

Ala Cys Gly Gly Asp Lys Pro Gly Val Arg Thr Val Tyr Leu Tyr Pro
                645                 650                 655

Asn Gln Thr Gly Leu Pro Asp Pro Leu Ser Arg His His Ala Phe Cys
            660                 665                 670

Phe Arg Gly Ile Ser Ala Val Pro Ser Pro Gly Glu Glu Glu Gly Gly
        675                 680                 685

Thr Pro Thr Ser Pro Ser Gly Val Glu Glu Trp Ile Val Thr Gln Val
    690                 695                 700

Val Pro Gly Val Ala Ala Val Pro Val Glu Glu Glu Thr Thr Ala Val
705                 710                 715                 720

Pro Ser Gly Glu Thr Thr Ala Ile Leu Glu Phe Thr Thr Glu Pro Glu
                725                 730                 735

Asn Gln Thr Glu Trp Glu Pro Ala Tyr Thr Pro Val Gly Thr Ser Pro
            740                 745                 750

Leu Pro Gly Ile Leu Pro Thr Trp Pro Pro Thr Gly Ala Glu Thr Glu
        755                 760                 765

Glu Ser Thr Glu Gly Pro Ser Ala Thr Glu Val Pro Ser Ala Ser Glu
    770                 775                 780

Glu Pro Ser Pro Ser Glu Val Pro Phe Pro Ser Glu Glu Pro Ser Pro
785                 790                 795                 800

Ser Glu Glu Pro Phe Pro Ser Val Arg Pro Phe Pro Ser Val Glu Leu
                805                 810                 815

Phe Pro Ser Glu Glu Pro Phe Pro Ser Lys Glu Pro Ser Pro Ser Glu
            820                 825                 830

Glu Pro Ser Ala Ser Glu Glu Pro Tyr Thr Pro Ser Pro Pro Glu Pro
        835                 840                 845

Ser Trp Thr Glu Leu Pro Ser Ser Gly Glu Glu Ser Gly Ala Pro Asp
    850                 855                 860

Val Ser Gly Asp Phe Thr Gly Ser Gly Asp Val Ser Gly His Leu Asp
865                 870                 875                 880

Phe Ser Gly Gln Leu Ser Gly Asp Arg Ala Ser Gly Leu Pro Ser Gly
                885                 890                 895

Asp Leu Asp Ser Ser Gly Leu Thr Ser Thr Val Gly Ser Gly Leu Thr
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           900                 905                 910

Val Glu Ser Gly Leu Pro Ser Gly Asp Glu Glu Arg Ile Glu Trp Pro
        915                 920                 925

Ser Thr Pro Thr Val Gly Glu Leu Pro Ser Gly Ala Glu Ile Leu Glu
    930                 935                 940

Gly Ser Ala Ser Gly Val Gly Asp Leu Ser Gly Leu Pro Ser Gly Glu
945                 950                 955                 960

Val Leu Glu Thr Ser Ala Ser Gly Val Gly Asp Leu Ser Gly Leu Pro
                965                 970                 975

Ser Gly Glu Val Leu Glu Thr Thr Ala Pro Gly Val Glu Asp Ile Ser
            980                 985                 990

Gly Leu Pro Ser Gly Glu Val Leu  Glu Thr Thr Ala Pro  Gly Val Glu
        995                 1000                1005

Asp Ile  Ser Gly Leu Pro Ser  Gly Glu Val Leu Glu  Thr Thr Ala
    1010                1015                1020

Pro Gly  Val Glu Asp Ile Ser  Gly Leu Pro Ser Gly  Glu Val Leu
    1025                1030                1035

Glu Thr  Thr Ala Pro Gly Val  Glu Asp Ile Ser Gly  Leu Pro Ser
    1040                1045                1050

Gly Glu  Val Leu Glu Thr Thr  Ala Pro Gly Val Glu  Asp Ile Ser
    1055                1060                1065

Gly Leu  Pro Ser Gly Glu Val  Leu Glu Thr Ala Ala  Pro Gly Val
    1070                1075                1080

Glu Asp  Ile Ser Gly Leu Pro  Ser Gly Glu Val Leu  Glu Thr Ala
    1085                1090                1095

Ala Pro  Gly Val Glu Asp Ile  Ser Gly Leu Pro Ser  Gly Glu Val
    1100                1105                1110

Leu Glu  Thr Ala Ala Pro Gly  Val Glu Asp Ile Ser  Gly Leu Pro
    1115                1120                1125

Ser Gly  Glu Val Leu Glu Thr  Ala Ala Pro Gly Val  Glu Asp Ile
    1130                1135                1140

Ser Gly  Leu Pro Ser Gly Glu  Val Leu Glu Thr Ala  Ala Pro Gly
    1145                1150                1155

Val Glu  Asp Ile Ser Gly Leu  Pro Ser Gly Glu Val  Leu Glu Thr
    1160                1165                1170

Ala Ala  Pro Gly Val Glu Asp  Ile Ser Gly Leu Pro  Ser Gly Glu
    1175                1180                1185

Val Leu  Glu Thr Ala Ala Pro  Gly Val Glu Asp Ile  Ser Gly Leu
    1190                1195                1200

Pro Ser  Gly Glu Val Leu Glu  Thr Ala Ala Pro Gly  Val Glu Asp
    1205                1210                1215

Ile Ser  Gly Leu Pro Ser Gly  Glu Val Leu Glu Thr  Ala Ala Pro
    1220                1225                1230

Gly Val  Glu Asp Ile Ser Gly  Leu Pro Ser Gly Glu  Val Leu Glu
    1235                1240                1245

Thr Ala  Ala Pro Gly Val Glu  Asp Ile Ser Gly Leu  Pro Ser Gly
    1250                1255                1260

Glu Val  Leu Glu Thr Ala Ala  Pro Gly Val Glu Asp  Ile Ser Gly
    1265                1270                1275

Leu Pro  Ser Gly Glu Val Leu  Glu Thr Thr Ala Pro  Gly Val Glu
    1280                1285                1290
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Glu Ile  Ser Gly Leu Pro Ser  Gly Glu Val Leu Glu  Thr Thr Ala
    1295                1300                1305

Pro Gly  Val Asp Glu Ile Ser  Gly Leu Pro Ser Gly  Glu Val Leu
    1310                1315                1320

Glu Thr  Thr Ala Pro Gly Val  Glu Glu Ile Ser Gly  Leu Pro Ser
    1325                1330                1335

Gly Glu  Val Leu Glu Thr Ser  Thr Ser Ala Val Gly  Asp Leu Ser
    1340                1345                1350

Gly Leu  Pro Ser Gly Gly Glu  Val Leu Glu Ile Ser  Val Ser Gly
    1355                1360                1365

Val Glu  Asp Ile Ser Gly Leu  Pro Ser Gly Glu Val  Val Glu Thr
    1370                1375                1380

Ser Ala  Ser Gly Ile Glu Asp  Val Ser Glu Leu Pro  Ser Gly Glu
    1385                1390                1395

Gly Leu  Glu Thr Ser Ala Ser  Gly Val Glu Asp Leu  Ser Arg Leu
    1400                1405                1410

Pro Ser  Gly Glu Glu Val Leu  Glu Ile Ser Ala Ser  Gly Phe Gly
    1415                1420                1425

Asp Leu  Ser Gly Val Pro Ser  Gly Gly Glu Gly Leu  Glu Thr Ser
    1430                1435                1440

Ala Ser  Glu Val Gly Thr Asp  Leu Ser Gly Leu Pro  Ser Gly Arg
    1445                1450                1455

Glu Gly  Leu Glu Thr Ser Ala  Ser Gly Ala Glu Asp  Leu Ser Gly
    1460                1465                1470

Leu Pro  Ser Gly Lys Glu Asp  Leu Val Gly Ser Ala  Ser Gly Asp
    1475                1480                1485

Leu Asp  Leu Gly Lys Leu Pro  Ser Gly Thr Leu Gly  Ser Gly Gln
    1490                1495                1500

Ala Pro  Glu Thr Ser Gly Leu  Pro Ser Gly Phe Ser  Gly Glu Tyr
    1505                1510                1515

Ser Gly  Val Asp Leu Gly Ser  Gly Pro Pro Ser Gly  Leu Pro Asp
    1520                1525                1530

Phe Ser  Gly Leu Pro Ser Gly  Phe Pro Thr Val Ser  Leu Val Asp
    1535                1540                1545

Ser Thr  Leu Val Glu Val Val  Thr Ala Ser Thr Ala  Ser Glu Leu
    1550                1555                1560

Glu Gly  Arg Gly Thr Ile Gly  Ile Ser Gly Ala Gly  Glu Ile Ser
    1565                1570                1575

Gly Leu  Pro Ser Ser Glu Leu  Asp Ile Ser Gly Arg  Ala Ser Gly
    1580                1585                1590

Leu Pro  Ser Gly Thr Glu Leu  Ser Gly Gln Ala Ser  Gly Ser Pro
    1595                1600                1605

Asp Val  Ser Gly Glu Ile Pro  Gly Leu Phe Gly Val  Ser Gly Gln
    1610                1615                1620

Pro Ser  Gly Phe Pro Asp Thr  Ser Gly Glu Thr Ser  Gly Val Thr
    1625                1630                1635

Glu Leu  Ser Gly Leu Ser Ser  Gly Gln Pro Gly Val  Ser Gly Glu
    1640                1645                1650

Ala Ser  Gly Val Leu Tyr Gly  Thr Ser Gln Pro Phe  Gly Ile Thr
    1655                1660                1665
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Asp Leu  Ser Gly Glu Thr Ser  Gly Val Pro Asp Leu  Ser Gly Gln
    1670                1675                1680

Pro Ser  Gly Leu Pro Gly Phe  Ser Gly Ala Thr Ser  Gly Val Pro
    1685                1690                1695

Asp Leu  Val Ser Gly Thr Thr  Ser Gly Ser Gly Glu  Ser Ser Gly
    1700                1705                1710

Ile Thr  Phe Val Asp Thr Ser  Leu Val Glu Val Ala  Pro Thr Thr
    1715                1720                1725

Phe Lys  Glu Glu Glu Gly Leu  Gly Ser Val Glu Leu  Ser Gly Leu
    1730                1735                1740

Pro Ser  Gly Glu Ala Asp Leu  Ser Gly Lys Ser Gly  Met Val Asp
    1745                1750                1755

Val Ser  Gly Gln Phe Ser Gly  Thr Val Asp Ser Ser  Gly Phe Thr
    1760                1765                1770

Ser Gln  Thr Pro Glu Phe Ser  Gly Leu Pro Ser Gly  Ile Ala Glu
    1775                1780                1785

Val Ser  Gly Glu Ser Ser Arg  Ala Glu Ile Gly Ser  Ser Leu Pro
    1790                1795                1800

Ser Gly  Ala Tyr Tyr Gly Ser  Gly Thr Pro Ser Ser  Phe Pro Thr
    1805                1810                1815

Val Ser  Leu Val Asp Arg Thr  Leu Val Glu Ser Val  Thr Gln Ala
    1820                1825                1830

Pro Thr  Ala Gln Glu Ala Gly  Glu Gly Pro Ser Gly  Ile Leu Glu
    1835                1840                1845

Leu Ser  Gly Ala His Ser Gly  Ala Pro Asp Met Ser  Gly Glu His
    1850                1855                1860

Ser Gly  Phe Leu Asp Leu Ser  Gly Leu Gln Ser Gly  Leu Ile Glu
    1865                1870                1875

Pro Ser  Gly Glu Pro Pro Gly  Thr Pro Tyr Phe Ser  Gly Asp Phe
    1880                1885                1890

Ala Ser  Thr Thr Asn Val Ser  Gly Glu Ser Ser Val  Ala Met Gly
    1895                1900                1905

Thr Ser  Gly Glu Ala Ser Gly  Leu Pro Glu Val Thr  Leu Ile Thr
    1910                1915                1920

Ser Glu  Phe Val Glu Gly Val  Thr Glu Pro Thr Ile  Ser Gln Glu
    1925                1930                1935

Leu Gly  Gln Arg Pro Pro Val  Thr His Thr Pro Gln  Leu Phe Glu
    1940                1945                1950

Ser Ser  Gly Lys Val Ser Thr  Ala Gly Asp Ile Ser  Gly Ala Thr
    1955                1960                1965

Pro Val  Leu Pro Gly Ser Gly  Val Glu Val Ser Ser  Val Pro Glu
    1970                1975                1980

Ser Ser  Ser Glu Thr Ser Ala  Tyr Pro Glu Ala Gly  Phe Gly Ala
    1985                1990                1995

Ser Ala  Ala Pro Glu Ala Ser  Arg Glu Asp Ser Gly  Ser Pro Asp
    2000                2005                2010

Leu Ser  Glu Thr Thr Ser Ala  Phe His Glu Ala Asn  Leu Glu Arg
    2015                2020                2025

Ser Ser  Gly Leu Gly Val Ser  Gly Ser Thr Leu Thr  Phe Gln Glu
    2030                2035                2040

Gly Glu  Ala Ser Ala Ala Pro  Glu Val Ser Gly Glu  Ser Thr Thr
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    2045                2050                2055

Thr Ser  Asp Val Gly Thr Glu  Ala Pro Gly Leu Pro  Ser Ala Thr
    2060                2065                2070

Pro Thr  Ala Ser Gly Asp Arg  Thr Glu Ile Ser Gly  Asp Leu Ser
    2075                2080                2085

Gly His  Thr Ser Gln Leu Gly  Val Val Ile Ser Thr  Ser Ile Pro
    2090                2095                2100

Glu Ser  Glu Trp Thr Gln Gln  Thr Gln Arg Pro Ala  Glu Thr His
    2105                2110                2115

Leu Glu  Ile Glu Ser Ser Ser  Leu Leu Tyr Ser Gly  Glu Glu Thr
    2120                2125                2130

His Thr  Val Glu Thr Ala Thr  Ser Pro Thr Asp Ala  Ser Ile Pro
    2135                2140                2145

Ala Ser  Pro Glu Trp Lys Arg  Glu Ser Glu Ser Thr  Ala Ala Asp
    2150                2155                2160

Gln Glu  Val Cys Glu Glu Gly  Trp Asn Lys Tyr Gln  Gly His Cys
    2165                2170                2175

Tyr Arg  His Phe Pro Asp Arg  Glu Thr Trp Val Asp  Ala Glu Arg
    2180                2185                2190

Arg Cys  Arg Glu Gln Gln Ser  His Leu Ser Ser Ile  Val Thr Pro
    2195                2200                2205

Glu Glu  Gln Glu Phe Val Asn  Asn Asn Ala Gln Asp  Tyr Gln Trp
    2210                2215                2220

Ile Gly  Leu Asn Asp Arg Thr  Ile Glu Gly Asp Phe  Arg Trp Ser
    2225                2230                2235

Asp Gly  His Pro Met Gln Phe  Glu Asn Trp Arg Pro  Asn Gln Pro
    2240                2245                2250

Asp Asn  Phe Phe Ala Ala Gly  Glu Asp Cys Val Val  Met Ile Trp
    2255                2260                2265

His Glu  Lys Gly Glu Trp Asn  Asp Val Pro Cys Asn  Tyr His Leu
    2270                2275                2280

Pro Phe  Thr Cys Lys Lys Gly  Thr Ala Thr Thr Tyr  Lys Arg Arg
    2285                2290                2295

Leu Gln  Lys Arg Ser Ser Arg  His Pro Arg Arg Ser  Arg Pro Ser
    2300                2305                2310

Thr Ala  His
    2315


<210> SEQ ID NO 2
<211> LENGTH: 12
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 2

Val Asp Ile Pro Glu Asn Phe Phe Gly Val Gly Gly
1               5                   10


<210> SEQ ID NO 3
<211> LENGTH: 12
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 3

Asn Ile Thr Glu Gly Glu Ala Arg Gly Ser Val Ile
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1 5 10

<210> SEQ ID NO 4
<211> LENGTH: 12
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 4

Ile Leu Thr Val Lys Pro Ile Phe Glu Val Ser Pro
1 5 10

<210> SEQ ID NO 5
<211> LENGTH: 12
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 5

Ala Phe Thr Ser Glu Asp Leu Val Val Gln Val Thr
1 5 10

<210> SEQ ID NO 6
<211> LENGTH: 12
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 6

Ala Phe Cys Phe Arg Gly Ile Ser Ala Val Pro Ser
1 5 10

<210> SEQ ID NO 7
<211> LENGTH: 10
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 7

Phe Phe Gly Val Gly Gly Glu Glu Asp Ile
1 5 10

<210> SEQ ID NO 8
<211> LENGTH: 10
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 8

Ala Arg Gly Ser Val Ile Leu Thr Val Lys
1 5 10

<210> SEQ ID NO 9
<211> LENGTH: 10
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 9

Ile Phe Glu Val Ser Pro Ser Pro Leu Glu
1 5 10

<210> SEQ ID NO 10
<211> LENGTH: 10
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 10

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Leu Val Val Gln Val Thr Ala Val Pro Gly
1 5 10

<210> SEQ ID NO 11
<211> LENGTH: 10
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 11

Ile Ser Ala Val Pro Ser Pro Gly Glu Glu
1 5 10

<210> SEQ ID NO 12
<211> LENGTH: 6
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 12

Val Asp Ile Pro Glu Asn
1 5

<210> SEQ ID NO 13
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 13

Phe Phe Gly Val Gly
1 5

<210> SEQ ID NO 14
<211> LENGTH: 7
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 14

Ala Arg Gly Ser Val Ile Leu
1 5

<210> SEQ ID NO 15
<211> LENGTH: 6
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 15

Ala Arg Gly Ser Val Ile
1 5

<210> SEQ ID NO 16
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 16

Phe Phe Gly Val Gly
1 5

<210> SEQ ID NO 17
<211> LENGTH: 5
<212> TYPE: PRT
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<400> SEQUENCE: 17

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Ala Arg Gly Ser Val
1 5

<210> SEQ ID NO 18
<211> LENGTH: 6
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 18

Asn Ile Thr Glu Gly Glu
1 5

1. A method of diagnosis of cardiovascular disease (CVD) comprising obtaining a patient biofluid sample, conducting an immunoassay to measure aggrecan fragments in said sample, and associating an elevation of said measure in said patient above a normal level with the presence of CVD, wherein said immunoassay is conducted by a method comprising:

contacting aggrecan fragments in said sample with an first immunological binding partner reactive with an N-terminal first epitope formed by cleavage of aggrecan by a proteinase and with a second immunological binding partner reactive with a second aggrecan epitope which is present in aggrecan at a location in the C-terminal direction from the location of said N-termainal epitope, and measuring the extent of simultaneous binding of aggrecan fragments to both said first and said second immunological binding partners to measure therein aggrecan fragments comprising both of said first and said second epitopes.

2. A method as claimed in claim **1**, further comprising comparing the measured amount of aggrecan fragments with a previously measured range of comparable values obtained for samples from a first group of patients having no cardiovascular disease and from a second group of patients previously diagnosed as having cardiovascular disease.

3. A method as claimed in claim **1**, wherein said first immunological binding partner has specific binding affinity for an N-terminal peptide sequence selected from the group consisting of:

Amino acid sequence	SEQ ID NO	Cleavage site location
*FFGVGGEEDI . . .	7	342
*ARGSVILTVK . . .	8	374
*IFEVSPSPLE . . .	9	
*LVVQVTAVPG . . .	10	
*ISAVPSPGEE . . .	11	

4. A method as claimed in claim **1**, wherein said second immunological binding partner has specific binding affinity for the G2 globular domain of aggrecan.

5. A method as claimed in claim **4**, wherein said second immunological binding partner has specific binding affinity for said globular domain of aggrecan even when said domain bears keratan sulphate chains.

6. A method as claimed in claim **1** or claim **2**, wherein said second immunological binding partner has specific binding affinity for keratan sulphate.

7. A method as claimed in claim **1**, wherein said immunoassay is conducted as a sandwich immunoassay.

8. A method as claimed in claim **1**, wherein said sample is a blood sample or a blood derived sample.

* * * * *

专利名称(译)	CVD风险评估的生化标志物		
公开(公告)号	US20100317023A1	公开(公告)日	2010-12-16
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摘要(译)

一种诊断心血管疾病（CVD）的方法，一种免疫测定法，用于测量所述样品中的聚集蛋白聚糖片段，以及高于正常水平的升高与CVD的存在的关联，通过使所述样品中的聚集蛋白聚糖片段与第一抗体反应而进行。通过蛋白酶切割聚集蛋白聚糖形成的N末端第一表位和与第二聚集蛋白聚糖表位反应形成的第二抗体，所述第二聚集蛋白聚糖表位存在于聚集蛋白聚糖中，位于所述N-末端表位的C-末端方向的位置，和测量两种抗体同时结合的程度。

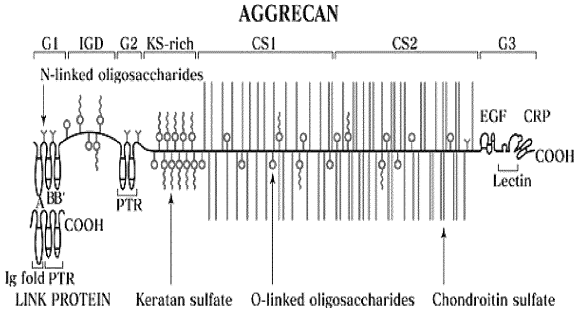

Figure 1