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(54) Title: JUNCTION PLAKOGLOBIN FOR DIAGNOSIS OF CARDIOVASCULAR DISEASES

(57) Abstract: The invention relates to a method for diagnosis of cardiovascular disease in a patient, com detecting, in a sample obtained from said patient, junction plakoglobin (SEQ ID 001) or a polypeptide related thereto. It further relates to devices and kits for detection of junction plakoglobin.

Junction Plakoglobin for diagnosis of cardiovascular diseases

Atherosclerosis is the leading cause of death worldwide. While fatty streaks develop into atheroma and then into complicated atherosclerotic plaques, the patient is often unaware of the risk and does not display any symptoms. Atherosclerosis underlies several diseases which limit the duration and quality of life, for example acute coronary syndrome (ACS) with acute myocardial infarction (AMI) being the most extreme form, stroke, peripheral arterial occlusion disease, renal artery stenosis, or aortic aneurysm. As yet atherosclerosis can be diagnosed either by the manifestation of its clinical complications in symptomatic patients or by imaging techniques. However these methods do only allow local diagnostics, thus providing surrogate information (e.g. measurement of carotid intima media thickness by Doppler sonography or NMR), are invasive (intravascular ultrasound), radiation-intensive (CT) and/or time consuming and expensive (CT and NMR).

Risk factors, combined in algorithms like the Framingham and PROCAM risk scores, help to identify asymptomatic individuals that are at high risk of developing coronary artery disease events. Risk factors include gender, age, family history, smoking, diabetes, obesity, blood pressure, triglycerides and total, HDL and LDL cholesterol. Algorithms provide a prediction for the occurrence of a cardiovascular event in the next 10 years. Although these clinical risk prediction rules have become an important component of international guidelines for the prevention of coronary artery disease (CAD), they are limited in sensitivity and specificity. Risk is overestimated in some populations and underestimated in others because it is modulated by non-quantifiable environmental and genetic factors.

In the last two decades, multiple novel biomarkers have been evaluated and although many of them show robust associations with CAD (for example C-reactive protein (CRP), troponins, or B-type natriuretic peptides), they do not improve the risk prediction by traditional markers significantly or with clinical relevance. The biomarkers tested as yet are either unspecific markers of inflammatory disease (e.g. CRP) or coagulation (e.g. fibrinogen), risk factors acting upstream of manifest disease (e.g. (apo-)lipoproteins), or downstream indicators of damages caused by atherosclerosis (e.g. troponins and B-type natriuretic peptides). In multi marker approaches, the downstream markers of structural and functional myocardial damage turned out as the most robust ones to improve risk prediction by conventional risk factors. At least in theory, biomarkers that reflect the atherosclerotic plaque burden of a patient may be of further use for risk prediction. If released by atherosclerotic plaques, its plasma concentration may correlate with the amount or severity of atherosclerosis.

There is a considerable medical need of soluble biomarkers for non-invasive, economical and high-throughput diagnostics of atherosclerosis. Such biomarkers may be helpful in i) improving risk

prediction and prognostics of cardiovascular and cerebrovascular diseases, ii) providing earlier and more reliable diagnosis of ACS and other arterial disease events such as stroke and rupture of aortic aneurysms, and iii) enabling to monitor progression and regression of atherosclerosis under treatment.

Hence, the objective of the present invention is to provide biomarkers for diagnosis of cardiovascular disease that improve on the state of the art. This objective is attained by the subject matter of the independent claims.

By using a hypothesis-free proteomics approach, it was surprisingly found that Junction Plakoglobin (JUP, SEQ ID 001) and the related proteins and peptides provided herein (in the following: *indicator proteins*) can serve as biomarkers of atherosclerosis. Atherosclerotic tissue and control tissue was excised from thromboendarterectomy material and incubated for 24 hours in protein-free culture medium. Released proteins were identified by a combination of subtractive antibody phage display screening and mass spectrometry. Immunohistochemistry of coronary thrombi and semiquantitative western blotting of plasma samples patients with or without coronary artery disease verified the diagnostic relevance of junction plakoglobin (JUP).

Particularly, an 81 kDa isoform corresponding to JUP, a 63 kDa isoform corresponding to cDNA FLJ60) as well as two anti-JUP-immunoreactive isoforms of 50 kDa and 30 kDa were identified as exemplary diagnostically valuable isoforms of Junction Plakoglobin.

Any known method may be used for measuring the levels of indicator proteins in a sample such as body fluids. Methods considered are e.g. chromatography, mass spectrometry (and combinations thereof), enzymatic assays, electrophoresis and antibody based assays (like ELISA, RIA, EIA, CEDIA, microarray analysis, fully-automated or robotic immunoassays and latex agglutination assays).

In the context of the present invention, the term “amount” also relates to concentration. From the total amount of a substance of interest in a sample of known size, the concentration of the substance can be calculated, and vice versa.

According to a first aspect of the invention, a method is provided for diagnosis of cardiovascular disease in a patient, or for assessing a patient’s risk of developing or having developed cardiovascular disease, said method comprising detecting an indicator protein selected from the group comprised of junction plakoglobin (SEQ ID 001), a polypeptide (SEQ ID 002) encoded by SEQ ID 004 (cDNAFLJ60), JUP peptide 1 (SEQ ID 007), JUP peptide 2 (SEQ ID 008), JUP peptide 3 (SEQ ID 009), JUP peptide 4 (SEQ ID 010), JUP peptide 5 (SEQ ID 011), JUP peptide 6 (SEQ ID 012) and JUP peptide 7 (SEQ ID 013). The method is preferably performed ex-vivo on a sample obtained from the patient.

According to one alternative to the first aspect of the invention, a method for classifying a sample obtained from a patient is provided, comprising the steps:

- detecting an indicator protein selected from the group comprised of junction plakoglobin (SEQ ID 001), a polypeptide (SEQ ID 002) encoded by SEQ ID 004 (cDNAFLJ60), JUP peptide 1 (SEQ ID 007), JUP peptide 2 (SEQ ID 008), JUP peptide 3 (SEQ ID 009), JUP peptide 4 (SEQ ID 010), JUP peptide 5 (SEQ ID 011), JUP peptide 6 (SEQ ID 012) and JUP peptide 7 (SEQ ID 013),
- assigning the sample to a probability of developing or having developed cardiovascular disease.

According to one embodiment of this aspect of the invention, the sample is a blood sample, preferably a peripheral blood sample. According to one embodiment of this aspect of the invention, the amount of the indicator protein is determined. According to one embodiment of this aspect of the invention, the amount of the indicator protein is determined and compared to a standard.

Modern bio-analytical methods facilitate the direct isolation and detection of proteins, i.e. without prior binding of the protein to an antibody or other selective ligand. The sample may be submitted, for example, to one or more runs of chromatography, followed by mass spectroscopy or multi-dimensional mass spectroscopy analyses (HPLC-MS; LC-MSMS), to identify the relevant protein component of a sample quantitatively. Sample preparation steps to remove, for example, cells or non-relevant blood components may precede the analysis.

A preferred embodiment of this first aspect of the invention includes the use of one or more ligands specifically reactive to junction plakoglobin (SEQ ID 001), a polypeptide (SEQ ID 002) encoded by SEQ ID 004 (cDNAFLJ60), JUP peptide 1 (SEQ ID 007), JUP peptide 2 (SEQ ID 008), JUP peptide 3 (SEQ ID 009), JUP peptide 4 (SEQ ID 010), JUP peptide 5 (SEQ ID 011), JUP peptide 6 (SEQ ID 012) or JUP peptide 7 (SEQ ID 013).

A ligand is “specifically reactive” to an indicator protein in the context of the present invention if the ligand binds to the indicator protein with a disassociation constant of 10^{-7}, 10^{-8} or 10^{-9} mole/l or less, but does not bind to control proteins. Control proteins are, by the way of non-limiting example, plasma proteins such as albumins, globulins, lipoproteins, fibrinogens, prothrombin, transferrin as well as related cellular proteins such as alpha and beta-catenin .

According to one embodiment of this aspect of the invention, the method further comprises the steps of contacting, in a first contacting step, the sample with a first ligand specifically reactive to the indicator protein, and determining, in a quantification step, the amount of the indicator protein bound to the ligand.

A ligand according to any aspect or embodiment of the invention may be any molecule that binds junction plakoglobin (SEQ ID 001), a polypeptide (SEQ ID 002) encoded by SEQ ID 004 (cDNAFLJ60), JUP peptide 1 (SEQ ID 007), JUP peptide 2 (SEQ ID 008), JUP peptide 3 (SEQ ID 009), JUP peptide 4 (SEQ ID 010), JUP peptide 5 (SEQ ID 011), JUP peptide 6 (SEQ ID 012) or JUP peptide 7 (SEQ ID 013) with high affinity and specificity.

High affinity in the sense of the invention refers to the dissociation constant of the binding of the ligand to junction plakoglobin (SEQ ID 001), a polypeptide (SEQ ID 002) encoded by SEQ ID 004 (cDNAFLJ60), JUP peptide 1 (SEQ ID 007), JUP peptide 2 (SEQ ID 008), JUP peptide 3 (SEQ ID 009), JUP peptide 4 (SEQ ID 010), JUP peptide 5 (SEQ ID 011), JUP peptide 6 (SEQ ID 012) or JUP peptide 7 (SEQ ID 013), wherein the dissociation constant is 10^{-7}, 10^{-8} or 10^{-9} mole/l or less.

High specificity in the sense of the invention refers to the ratio of properly detected junction plakoglobin (SEQ ID 001), a polypeptide (SEQ ID 002) encoded by SEQ ID 004 (cDNAFLJ60), JUP peptide 1 (SEQ ID 007), JUP peptide 2 (SEQ ID 008), JUP peptide 3 (SEQ ID 009), JUP peptide 4 (SEQ ID 010), JUP peptide 5 (SEQ ID 011), JUP peptide 6 (SEQ ID 012) or JUP peptide 7 (SEQ ID 013) and the sum of all detected polypeptides, wherein the ration is 80%, 85%, 90%, 95%, 99% or 99,9%.

In one embodiment, the ligand is an antibody. In one embodiment, the ligand is a gamma immunoglobulin. In one embodiment, the ligand is a monoclonal antibody binding to or raised against JUP.

In one embodiment the ligand is a single-domain antibody, for instance a single-chain variable fragment (scFv) antibody selected by phage display methods, or a camelide antibody, specifically reactive to the indicator protein.

Alternatively, a ligand employed in practising the invention may be a molecule engineered or selected to demonstrate a high specificity and avidity for the indicator protein. Such engineered or selected molecule may be a antibody-like polypeptide or synthetic antibody selected by phage-display (see Pini et al., J.Biol. Chem. (1998) 273, 21769-21776), or by other evolutionary selection methods. Alternatively, the ligand may be a polynucleotide or spiegelmer with high selectivity for the indicator protein (e.g. SELEX, see Ellington & Szostak, *Nature* 346, 818-822).

According to a preferred embodiment of this aspect of the invention, a single chain antibody comprising the amino acid sequence of Seq ID 005 or encoded by the nucleic acid sequence of Seq ID 006 is employed as a ligand.

In some embodiments, the method according to the invention will involve the comparison of the determined amount of protein with a standard.

In some embodiments, an absolute measurement may achieve the desired accuracy and validity, for example if a reproducible determination of binding can be attained, as could be the case for surface plasmon resonance (SPR) assays with a properly calibrated SPR device, or spectroscopic read-outs in HPLC, where the integral of the chromatogram is proportional to the amount of analyte.

According to one embodiment of this aspect of the invention, the ligand has a fluorescent or luminescent quality and the determining is performed by fluorescence-activated cytometry. A

fluorescent or luminescent quality in the sense of the invention refers to the ability of the first ligand to emit light after excitation.

According to one embodiment, the ligand is attached to a surface. A preferred surface is the surface of a microtiter well or a lab-on-a-chip device (LOC, microfluidics analysis).

A ligand is attached, in the context of the present invention, if the ligand is bound to the surface by covalent or non-covalent bonds so that the attachment withstands repeated washing steps with aqueous buffer of pH 5 - 8. One example is a covalent bond, for example by ester or amide linkage; another is the streptavidin-biotin bond or simple adsorption of proteins to polymer surfaces. Ligands may be attached to gold surfaces by sulfide groups.

In one embodiment the surface is the surface of a plasmon resonance or quartz microbalance chip, which will allow direct measurement of the amount of indicator protein attached to the ligand by means of the changes effected in the physical properties of the chip. The amount of indicator protein is thus determined directly.

According to one embodiment of the first aspect of the invention in any of the embodiments described herein further comprises, as part of the quantification step, the use of a second ligand with specificity for the targeted indicator proteins. The second ligand binds to a different surface section of the target in order to allow for the first and second ligand to be able to bind concomitantly.

The second ligand may carry an enzymatic activity or label, such as a peroxidase, to allow for fitting the method into the well-established ELISA formats.

Likewise, the second ligand may have an optically detectable quality such as a colour label or a fluorescent or luminescent quality.

In general, suitable labels are chromogenic labels, i.e. enzymes which can be used to convert a substrate to a detectable colored or fluorescent compound, spectroscopic labels, e.g. fluorescent labels or labels presenting a visible color, affinity label which may be developed by a further compound specific for the label and allowing easy detection and quantification, or any other label used in standard ELISA.

An optically detectable label or enzyme activity may be detected on a surface.

According to one embodiment, the method according to the invention thus comprises the following steps:

- in a first contacting step, a blood or other fluid sample obtained from a patient is contacted, optionally after a work-up for removal of irrelevant components, with a surface onto which a first ligand specifically reactive to the indicator protein is attached;
- in a second contacting step, a second ligand is contacted with the surface, the sample is removed along with any component previously contained therein that has not been bound

by the first ligand, and the amount of the second ligand is determined.

- Optionally, a washing step is inserted between the first and the second step to remove any sample components other than the indicator protein. Another washing step may follow the second contacting step and precede the determination.

According to one embodiment, the first and the second ligand are attached to the respective components of a system that allows to determine a quantitative measure of proximity-paired partners, such as the “Amplified Luminescent Proximity Homogeneous Assay (ALPHA)” commercialized by Perkin Elmer, whereby singlet oxygen transferred from a donor partner to an acceptor, resulting in emission of light, if the two partners are within 200nm distance. Such arrangement allows for the practice of the invention in solution.

According to one embodiment of this aspect of the invention, a liquid volume of sample is placed onto a sample area composed in such way as to provide capillary forces that act to transport at least a part of the sample towards an analysis area. Between the sample area and the analysis area, or as part of the sample area, a conjugate area is provided. First ligands specific for the indicator protein are provided, without attachment to the matrix, in the conjugate area, and upon binding to indicator protein in the sample, these first ligands are drawn with the capillary flow into the analysis area. The first ligands according to this embodiment of the invention are conjugated to latex or gold microparticles or enzymatically active proteins. Second ligands are attached to the analysis area, forming a line. Upon binding of indicator proteins (with first ligands attached) to the second ligands, the resulting “sandwiches” of second ligand, indicator protein and first ligand form a line, which may be visible as such (if particles are attached to the first ligands), or may be rendered visible by providing a substrate for an enzymatic activity attached to the first ligand. This embodiment is referred to as a lateral flow assay.

According to a preferred embodiment of any of the method aspects of the present invention, the level of indicator protein(s) in the sample is compared to the corresponding levels in (a) sample(s) of (a) healthy subject(s). An altered level compared to the level in the sample of the healthy subject is indicative of a disease or of the risk to develop the disease.

According to this preferred embodiment of the present invention, the measured levels of indicator proteins indicates whether an individual is suffering from a cardiovascular disease or has an increased risk of developing such disease. The terms used in this context, i.e. "non-increased level", “not elevated level”, "increased levels” and “decreased levels” are known to, or can be determined by the person skilled in the art according to, for example, the procedure outlined in the following paragraphs.

The person skilled in the art is able to determine actual values corresponding to "non-increased level", “not elevated level”, "increased levels” and “decreased levels” for the relevant biochemical markers. For example, the levels may be assigned according to percentiles of the levels observed

in a representative sample of apparently healthy individuals, typically below an age of 50 years. The sample should be of sufficient size to yield statistically significant outcomes (for example, at least 100, more preferably at least 500, most preferably at least 1000 individuals). A non-increased level may correspond, by way of example, to the maximum level observed in the 97.5% percentile of healthy individuals. Alternatively, the levels may be determined as "normal ranges" as known in the state of the art.

The levels may also be determined or further refined by studies performed on individuals by comparing levels of the indicator proteins of apparently healthy individuals with individuals suffering from a cardiovascular disease. Such studies may also allow to tailor the levels according to the type of disease or/and certain patient sub-groups, e.g. elderly patients, patients undergoing medication or patients with a certain lifestyle. Guidance on how such studies may be carried out can also be obtained from the examples as disclosed in this specification.

The value of the levels considered as "increased" or “decreased” may also be chosen according to the desired sensitivity or specificity (stringency) of exclusion. The higher the desired sensitivity, the lower is the specificity of exclusion and vice versa. In the above example, the higher the percentile chosen to determine each level, the more stringent is the exclusion criterion, i.e. less individuals would be considered "risk individuals".

The method according to the present invention also allows the determination of the risk or the likelihood, respectively, of an individual of suffering from a cardiovascular disease. In the context of the present invention, the terms “risk” or “likelihood” relates to the probability of a particular incident to develop a cardiovascular disease. The grade of risk can be decreased, non-increased, increased, or highly increased. “Non-increased risk” or “no likelihood” means that there is apparently no risk of suffering from or of developing a cardiovascular disease.

The degree of risk is associated with the levels of indicator proteins. A non-altered level of indicator proteins indicates no increased risk, an altered level of indicator proteins indicates an increased risk, and a highly altered level of indicator proteins indicates a highly increased risk.

If the level of indicator proteins is altered, the patient will undergo a primary treatment or secondary interventions like the initiation of therapeutic life-style changes, drug therapy depending on risk factor constellation and reviews at regular intervals. If the methods according to the present invention indicate an increased or highly increased risk, it will preferably have consequences for the further treatment of the individual.

Hence, the term “diagnosis” in the context of the present invention includes (by non-limiting example) diagnosis of acute disease, determination of risk to develop or to be diagnosed with acute disease, and monitoring of the state of disease during or after treatment.

According to a second aspect of the invention, a surface is provided that comprises a ligand specifically reactive to an indicator protein selected from the group comprised of junction

plakoglobin (SEQ ID 001), a polypeptide (SEQ ID 002) encoded by SEQ ID 004 (cDNAFLJ60), JUP peptide 1 (SEQ ID 007), JUP peptide 2 (SEQ ID 008), JUP peptide 3 (SEQ ID 009), JUP peptide 4 (SEQ ID 010), JUP peptide 5 (SEQ ID 011), JUP peptide 6 (SEQ ID 012) and JUP peptide 7 (SEQ ID 013). Such surface may be an ELISA plate, a LOC device, or the matrix material of a lateral flow immunoassay.

According to a third aspect of the invention, a device for diagnosis of cardiovascular disease is provided comprising a ligand specifically reactive to an indicator protein selected from the group comprised of junction plakoglobin (SEQ ID 001), a polypeptide (SEQ ID 002) encoded by SEQ ID 004 (cDNAFLJ60), JUP peptide 1 (SEQ ID 007), JUP peptide 2 (SEQ ID 008), JUP peptide 3 (SEQ ID 009), JUP peptide 4 (SEQ ID 010), JUP peptide 5 (SEQ ID 011), JUP peptide 6 (SEQ ID 012) and JUP peptide 7 (SEQ ID 013).

According to a preferred embodiment of this aspect of the invention, the ligand is attached to a surface, preferably the surface of a LOC device, a surface plasmon resonance chip, a quartz microbalance and a lateral flow assay device.

According to yet another aspect of the invention, a kit of parts is provided for diagnosis of cardiovascular disease in a patient, or for assessing a patient's risk of developing or having developed cardiovascular disease, said kit comprising a first ligand and a second ligand, both ligands being specifically reactive to an indicator protein selected from the group comprised of Junction plakoglobin (SEQ ID 001), a polypeptide (SEQ ID 002) encoded by SEQ ID 004 (cDNAFLJ60), JUP peptide 1 (SEQ ID 007), JUP peptide 2 (SEQ ID 008), JUP peptide 3 (SEQ ID 009), JUP peptide 4 (SEQ ID 010), JUP peptide 5 (SEQ ID 011), JUP peptide 6 (SEQ ID 012) and JUP peptide 7 (SEQ ID 013).

According to a one embodiment of this aspect of the invention, the first ligand is attached to the surface of a microtiter plate, and the second ligand comprises an enzymatic activity or a luminescent activity.

In some embodiments, the ligands are monoclonal antibodies or single chain antibodies.

In one embodiment, the ligand is a single chain antibody comprising the amino acid sequence of Seq ID 005 or being encoded by the nucleic acid sequence of Seq ID 006.

According to one aspect of the invention, a surface or a device according to the above aspects or embodiments is provided for use in a method according to the above aspects or embodiments of the invention.

Wherever reference is made herein to an embodiment of the invention, and such embodiment only refers to one feature of the invention, it is intended that such embodiment may be combined with any other embodiment referring to a different feature.

The invention is further characterized, without intent to limit its scope, by the following examples,

from with further features, advantages or embodiments can be derived. The examples do not limit but illustrate the invention.

Brief description of the figures

Fig. 1 shows a schematic overview of the approach used to identify potential markers of atherosclerosis;

Fig. 2 shows immunoblotting experiments to detect potential markers of atherosclerosis in secretomes;

Fig. 3 shows the amino acid sequences of JUP (A) and of the JUP variant encoded by cDNA FLJ60424 (B);

Fig. 4 shows results of experiments to determine the plasma levels of JUP in patients with ACS, patients with stable CAD and healthy controls by A) Detection of JUP by immunoblotting. and B) by semi-quantitative analysis of western blots;

Fig. 5 shows the detection of JUP in thrombi by immunohistochemistry;

Fig. 6 shows the detection of JUP in thrombi by immunoblotting.

Fig. 7 shows the detection of JUP in monocytes and differentiated macrophages.

Fig. 8 shows the detection of JUP isoforms in plasmas of mice with or without atherosclerosis.

Fig. 9 shows the detection of JUP isoforms in plasmas of patients with peripheral atherosclerotic vascular disease (PAVK).

The findings on which the present invention is based provide three lines of evidence that JUP may serve as a biomarker of atherosclerosis: Firstly, secretomes of atherosclerotic arteries but not healthy arteries contain proteins which immunoreact with both the phage display antibody scFv 25G5 used in determining the relevance of JUP, and with independent commercial anti-JUP-antibodies. Secondly, plasma levels of JUP were increased 2.4-fold in patients with stable CAD and 14.1-fold in patients with ACS, compared to healthy controls. Thirdly, monocytes/macrophages as well as the cell-free parts of the fibrin clots of coronary thrombi of ACS patients show strong anti-JUP-immunoreactivity. In addition, anti-JUP-immunoreactive proteins of 63 kD, 55 kD and 30 kD were detected in atherosclerotic secretomes, lysates of coronary thrombi and macrophages that were differentiated *in vitro* from peripheral blood monocytes. The 55kD and 33 kD isoforms were also found in plasmas of mice with atherosclerosis and patients with peripheral atherosclerotic vascular disease (figures 8 and 9).

JUP is a protein component of desmosomes, which are junction complexes with essential structural functions in tissues that experience mechanical stress. Desmosomes connect neighbouring cells to each other through their transmembrane cadherins (desmocollin and

desmoglein). The cytoplasmic tails of cadherins are, indirectly through JUP, plakophilin and desmoplakin, connected to the intermediate filaments. The essential physiological role of JUP for regular functionality of desmosomes in the myocardium is indicated by the findings of premature cardiac death of JUP knock-out (-/-) mice and arhytmogenic right ventricular cardiomyopathy in patients carrying functionally relevant mutations in the JUP gene. The importance of JUP for endothelial integrity is indicated by the results of several in vitro experiments. In neovessels of atherosclerotic arteries JUP only becomes associated with cell-to-cell junctions of near confluent cells, suggesting that JUP is required for the formation of mature cytoskeleton-bound junctions. On the contrary leukocyte adhesion to confluent TNFα-activated endothelial monolayers induces dissociation of JUP and beta-catenin from vascular endothelial (VE)-cadherin, possibly as a prerequisite for diapedesis of the blood borne cells. The finding of increased JUP plasma levels in ACS and CAD patients can hence reflect both myocardial and endothelial damage. Although the myocardial origin of JUP cannot be ruled out at present, the finding of strong anti-JUP immunoreactivity in coronary thrombi of ACS patients strongly indicates the contribution of vascular origin.

In addition to the 82 kD JUP, a 63 kD JUP-homologue which is encoded by cDNA FLJ60424, was identified by means of both mass spectrometry and western blotting of atherosclerotic secretomes. Both proteins were immunoprecipitated from secretomes with scFv 25G5. Either scFv 25G5 recognizes an epitope that is present on the N-terminal part that is identical in both proteins, or the epitope of scFv 25G5 is located specifically on the 63 kD JUP variant, which then binds the 82 kD JUP in the atherosclerotic plaque secretome, e.g. by forming a dimer or supramolecular complex so that the 82 kD JUP protein and the 63 kD variant are co-immunoprecipitated. Currently, nothing is known about this protein, since it has only been described as a coding DNA submitted in the EMBL/GenBank/DDBJ databases. Cloning and expression of both the 82 kD intact JUP protein and the 63 kD JUP variant in bacterial and mammalian cells will firstly provide more insight into the location of the epitope that is recognized by 25G5, and secondly allow the generation of antibodies and standards for sandwich ELISAs to quantify these proteins in patient samples.

Two additional protein bands of 55 and 30 kD were both detected with relatively high immunoreactivity in atherosclerotic secretomes, lysed thrombi and macrophages that were differentiated *in vitro* from peripheral blood monocytes, with the 30 kD band being weakly detectable in plasma as well. The 55kD and 33 kD isoforms were also found in plasmas of mice with atherosclerosis and patients with peripheral atherosclerotic vascular disease (figures 8 and 9). These are likely to be degradation products of intact JUP and/or of the 63 kD variant, or alternative splice variants of JUP which may also have high utility as clinical markers of cardiovascular disease.

Currently, no plasma biomarker for coronary artery disease exists that is continuously released from atherosclerotic plaques and thus represents the atherosclerotic plaque load. The presence of

atherosclerosis can only be measured by imaging techniques that are time-consuming, expensive and not suitable for screening large populations or large clinical trials. JUP and the described variants of 63, 55 and 30 kD might be the first blood biomarkers that can be used as a measure of plaque load and will be a large step forward in clinical diagnostics of cardiovascular diseases. Studies with larger patient cohorts, using immunoassays that are under development, will validate the application of JUP and the smaller JUP variants as diagnostic, prognostic and monitoring markers of vascular diseases.

The present invention may be used for assessing the risk of patients for developing clinical complications of atherosclerosis, i.e. myocardial infarction or stroke. In principle, biomarkers released by atherosclerotic plaques are the closest indicators of existing disease and hence good candidates to improve risk prediction. Compared to conventional risk factors such a marker of existing disease should also allow more reproducible absolute risk prediction in different populations than risk factors of which the penetrance is strongly influenced by environmental factors.

The means and methods provided herein may be used for monitoring the progression and regression of atherosclerotic disease upon treatment. As yet progression and regression of CAD can only be assessed by imaging techniques. In addition, biomarkers of atherosclerosis may open the avenue to monitor treatment success by simple blood testing. The availability of such monitoring blood test will be helpful not only for clinical management of patients but also for novel drug development because such biomarker may be used as surrogate marker in phase 3 trials without clinical endpoints.

If the biomarker used herein (the indicator protein) is released only by rupturing or eroding plaques, which eventually cause acute clinical events (acute coronary syndrome, stroke) it will also be helpful in the acute diagnostics of myocardial infarction. As yet this diagnosis is possible either because of changes in the electrocardiogram, which however occur in only 30 to 50% of patients with myocardial infarction (ST-elevation myocardial infarction), or by assessing the elevated concentrations of cardiac troponins I or T in the blood. This biomarker is sensitive only at a minimum of 3 hours after the onset of symptoms.

Measuring the presence of indicator proteins in the blood is a cheap, fast and non-invasive alternative. No biomarkers are currently available that are released from atherosclerotic plaques and therefore are a measure of the plaque load. Hence, the present invention is not only useful to identify people at risk but also to monitor interventions.

Examples

Using the approach depicted in Figure 1, a list of potential biomarkers was generated, which included junction plakoglobin (JUP). The pictograms signify (top to bottom): 1. Preparation of secretomes; 2. subtractive phage display to select binders against proteins that are only present in

artherosclerotic secretome; 3. Screening > 500 monoclonal antibodies in ELISA; 4. Further screening of 34 selected scFV antibodies (ELISA on other secretomes, phage western blotting, scFV expression, scFV cDNA sequencing); 5, IP/MS with 9 selected scFV antibodies; 6. Analysis and priorization of identified proteins; 7. Validation of JUP in secretomes and plasma by immunoblotting, and in tissues by immunohistochemistry; 8. Direct MS analysis of atherosclerotic plaque and control secretomes).

In short, after several subtractive antibody phage display selections on different atherosclerotic secretomes, more than 500 monoclonal antibodies were screened for specific reactivity with atherosclerotic secretomes. Finally, nine antibodies were chosen for target identification, because they were expressed at good quantities and showed specific and relatively strong reactivity with atherosclerotic secretomes from more than one patient. The scFvs were used to immunoprecipitate their target antigens from atherosclerotic and control secretomes, and these isolated proteins were subsequently identified by mass spectrometry (IP/MS). After correction for non-specific background binding to the magnetic beads and an unrelated scFv antibody, a total of 105 proteins were identified to be precipitated by these nine antibodies. Out of these 105 proteins, 27 were exclusively detected by immunoprecipitation but not by direct proteomics analysis of the secretomes with two of these 27 being JUP (Swissprot ID P14923) and cDNA FLJ60424 (Swissprot ID B4DE59).

JUP is a protein with a known molecular weight of 82 kD, which could be weakly detected in atherosclerotic secretomes by the use of a commercially available mouse monoclonal anti-JUP-antibody (mAb 2C9) (Figure 6). Much stronger was the reactivity of 2C9 with two proteins running at approximately 30 and 55 kD (see Figure 2A), which may represent degradation products, alternative splice variants, or homologues of JUP. Both proteins were specifically detected in atherosclerotic secretomes but not in control secretomes. These two bands of 30 kD and 55 kD were also detected in the same secretomes by scFv 25G5 (see Figure 2B), the scFv by which JUP was initially identified. ScFv 25G5 additionally reacted with a protein of approximately 65 kD, which was not detected by the commercial antibody. The 65 kD protein represents a protein which is encoded by cDNA FLJ60424 (Swissprot ID B4DE59). It is highly homologous to JUP and has a predicted molecular weight of 63 kD. It was also identified in the IP/MS experiment upon immunoprecipitation with scFv 25G5. Figure 3 depicts the amino acid sequences of the intact 82 kD JUP protein and of the 63 kD JUP variant, as well as the peptides that led to the protein identifications in the IP/MS experiment. Four peptides were detected that can be assigned to both intact JUP and to the smaller variant, four peptides were identified that are specific for the 63 kD JUP variant and two peptides were found that are only present in intact JUP.

As depicted in Figure 4A and Figure 8, the 82 kD intact JUP protein could be clearly detected in plasma samples, which were depleted of albumin and IgG, by immunoblotting with the commercial anti-JUP mAb 2C9. Plasma levels of JUP were analyzed in patients with ACS (n = 11), patients

with stable CAD (n = 15) and healthy, angiographically confirmed CAD-free controls (n = 13). An overview of the patient characteristics is presented in Table 1. Two plasma samples, one with a relatively high JUP concentration and one with a relatively low JUP concentration, were run on each blot for standardization. The intensities of the JUP bands running at 82 kD were quantified in all samples with ImageJ (National Institutes of Health) and were then expressed as relative intensities compared to the intensity of the two controls. With this semi-quantitative approach, using the low JUP concentration as a reference on all blots, JUP plasma levels were found 2.4-fold elevated in CAD patients compared to controls (p=0.036), and even 14.1-fold elevated in ACS patients when compared to controls (p = 0.021) (Figure 4B).

JUP-reactive bands were also identified in patients with peripheral artery occlusive disease (PAOD).

Example 1: Detection of JUP and the smaller JUP variant, encoded by cDNA FLJ60424, in secretomes by immunoblotting.

Proteins from six atherosclerotic plaque secretomes (Figure 2, lanes 1 - 6), from two control secretomes (lanes 7 and 8), as well as recombinant, GST-tagged JUP (lane 9) were separated on 12.5% polyacrylamide gels and transferred to nitrocellulose membranes for detection. A) Reactivity of a commercial anti-JUP mAb. In lane 9, 50 ng GST-tagged JUP protein (107 kDa), was used as positive control and is clearly detected by the anti-JUP antibody. In three plaque secretomes bands of around 55 and 30 kD are detected. B) Reactivity of the affinity-selected scFv antibody 25G5. The scFv 25G5 does not detect 500 ng recombinant GST-tagged JUP protein used as a positive control (lane 9). Strong reactivity with a protein band of approximately 65 kD, as well as reactivity with protein bands of around 55 and 30 kD was observed.

Example 2: Amino acid sequences.

The sequences of JUP (A) and of the JUP variant encoded by cDNA FLJ60424 (B) are shown in Fig. 3. The N-terminal part (bold) is identical in both proteins. Peptides that were found in the IP/MS experiment in which scFv 25G5 was used as bait are indicated. The peptides marked by underlining are found in both proteins, the peptides in italics (dotted underlining) are specific for JUP and the peptides marked in italics are only present in the JUP variant that is encoded by cDNA FLJ60424.

Example 3: Plasma levels of JUP in patients with ACS, patients with stable CAD and healthy controls.

Figure 4.A) shows detection of JUP by immunoblotting: recombinant GST-tagged JUP (107 kD, lane 1) was used as a positive control. Plasma samples were run in duplicates on SDS-PAGE gels, transferred to nitrocellulose membranes and probed with anti-JUP mAb 2C9. The 82 kD JUP protein was detected in a control plasma (lanes 2 and 3), in plasma from a CAD patient (lanes 4 and 5) and in plasma from an ACS patient (lanes 6 and 7). An additional protein band was

detected around 30 kD. B) Semi-quantitative analysis of western blots. In order to compare results from different western blots, one plasma sample was run on each blot and the intensity of the JUP band of this plasma was used to correlate the intensities of the JUP bands in the plasma samples (relative intensity meaning relative to JUP band in the control plasma). JUP plasma levels were measured in 11 ACS patients, 15 CAD patients and 13 healthy controls.

Example 4: Detection of JUP in thrombi by immunohistochemistry. Figure 5 shows thrombi from two patients (patient 1: upper pictures, and patient 2: lower pictures) that were stained with the commercial anti-JUP mAb 2C9 (A and C). Negative control stainings, representing reactivity of the secondary antibody only, do not show any reactivity (B and D). Cells in which JUP was detected are most likely monocytes and macrophages. In addition, a strong extracellular staining was observed.

Example 5: Detection of JUP in thrombi by immunoblotting. JUP was detected on western blot containing recombinant GST-tagged JUP (Figure 6, lane 1, 107 kD), secretome from an atherosclerotic plaque (lane 2), plasma from an ACS patient (lane 3) and lysates of two thrombi (lanes 4 and 5) with anti-JUP mAb 2C9. A band of 55 kD is strongly detected in secretome and in lysates of the two thrombi. Intact JUP (82 kD) is detected in plasma, as well as in secretome, but not in thrombi. A protein band of 30 kD is detected in secretome and in plasma. In secretome, a band of slightly higher molecular weight is detected.

Example 6: Detection of JUP in monocytes and differentiates macrophages. Monocytes were isolated from buffy coat preparations (peripheral blood enriched for leukocytes and platelets) using magnetic CD14 beads and grown in cell culture medium for nine days, during which the monocytes differentiated spontaneously into macrophages. Cells were harvested, lysed and proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. JUP isoforms were detected with anti-JUP monoclonal antibody 2G9 (replacement of 2C9, Fig. 7). Recombinant GST-tagged JUP-81 (Fig. 7, lane 1), plaque secretome (Fig. 7, lane 2) and thrombus lysates (Fig. 7, lane 3) were loaded as positive controls for different JUP isoforms. Monocytes (day 0, Fig. 7, lane 4) and differentiated macrophages harvested on day 2 (Fig. 7, lane 5), day 5 (Fig. 7, lane 6), day 7 (Fig. 7, lane 7) and day 9 (Fig. 7, lane 8) demonstrated increasing JUP-55 (55kDa) and JUP-30 (30 kDa) concentrations.

Example 7: JUP isoforms in plasmas of mice with or without atherosclerosis

Figure 8 shows JUP in plasma samples of mice with or without atherosclerosis. Recombinant JUP (lane 1) and JUP isoforms of plaque secretome (lane 2), plasma of an ACS patient (lane 3), wild-type mice (lanes 4 and 5) and LDL-receptor KO mice transplanted with bone marrow of ABCG1 x apoE double KO mice (lanes 6 and 7) or ABCA1 KO mice (lanes 8 and 9) were visualized by immunoblotting using a polyclonal rabbit antibody against JUP. Note the presence of the anti-JUP-immunoreactive isoform with 55 kD molecular weight in plasmas of mice with atherosclerosis but

not without.

Example 8: JUP isoforms in plasmas of patients with peripheral atherosclerotic vascular disease (PAVK), ACS and healthy controls.

Figure 9 shows detection of JUP by immunoblotting: recombinant GST-tagged JUP (107 kD, lane 2) was used as a positive control, lanes 3 and 4 contain plasmas of a healthy control individual and an ACS patient, respectively. Lanes 5 through 9 contain plasmas of five different patients with peripheral atherosclerotic vascular disease (PAVK). The normal JUP isoform JUP81 as well as a small molecular weight isoform (JUP31) are present in every sample, however at varying amounts. Interestingly JUP55 occurs only in samples of patients with PAVK. The experiment was performed as described in figure 4a

Materials and Methods

The tissue material and plasma samples investigated in this study were obtained after approval by the Ethical Committees in Zurich and Basel, respectively, and by informed consent of the donating patients.

Secretomes

Specimen of arteries were obtained by thromboendarterectomy of carotis or iliaca at the Bruderholz Hospital Basel and the plaque part was graded on a scale from 1 to 3, with 1 representing initial stage lesions equivalent to Stary Type I – III, 2 representing medium stage lesion equivalent to Stary Type IV and V, and 3 representing advanced stage complicated lesion equivalent to Stary Type VI - VIII . The best preserved part was healthy (below Stary Type 1) in all tissue samples used. Plaque and control tissue sections were cut into similar size pieces of about 3 mm x 6 mm, washed with phosphate buffered saline (PBS) and individually incubated for 24 hours at 37°C in protein-free RPMI medium (RPMI-1640, Sigma Aldrich) containing 100 µg/ml ampicillin (Merck). After the incubation time, the media containing the secreted and washed out proteins were collected, centrifuged and stored at -20°C until further use. The secretome proteins were biotinylated using EZ-Link Sulfo-NHS-LC-Biotin (Pierce, Thermo Scientific). Consistency of biotinylation was confirmed on western blot.

Plasma samples and thrombi

Male subjects aged 54 to 65 admitted to the cardiac catheterisation laboratory at the University Hospital Zurich between December 2009 and October 2010 for coronary angiography either for suspected coronary artery disease, follow-up, or preoperatively before valve replacement or bypass surgery, were included in the study. Patients with a history of ACS less than six months prior to the study as well as individuals suffering from an active neoplastic, infectious or autoimmune disease were excluded. Blood parameters were measured on automated routine analysers. Coronary thrombi were aspirated from the site of coronary occlusion during primary

percutaneous coronary intervention (PCI) in patients with ACS using an Exportw XT 6F Aspiration Catheter (Medtronic Inc., Minneapolis, MN, USA). The harvested thrombi were separated from blood with a sieve, placed in phosphate buffered saline (PBS), and immediately processed The solid material was kept in buffered paraformaldhyde 4% for 4-6 hours and in 50% ethanol for 24 hours prior to paraffin embedding and xylol/ethanol fixation and cutting with a microtome.

Antibody selections

The large synthetic human single chain variable fragment (scFv) phage display library ETH-2-Gold, containing 3 billion individual antibody clones, was amplified as described (Silacci et al., (2005) *Proteomics* **5**, 2340-2350). Prior to selection, binders against some of the common plasma proteins, namely apo-transferrin, holo-transferrin, immunoglobulin G (purified from serum using Protein G Dynabeads), fibrinogen and complement component 3, were removed. Six selections were done with two subtractive selection rounds each. Each selection was performed on plaque and control secretomes from a single patient. Subtraction was achieved by first removing phages binding to control secretome (i.e. secretome produced with the best preserved part of the tissue). The remaining, unbound phages were subsequently panned against the biotinylated plaque secretome from the same patient, while competing with an excess of non-biotinylated control secretome. Bound phages were collected using magnetic streptavidin-coated beads. Unbound phages were washed off, and bound phages were eluted and amplified. Polyclonal phage pools of each round were screened in an enzyme-linked immunosorbent assay (ELISA) for reactivity with control and plaque secretome.

Screening and characterization of single clones

Single colonies were picked into two round-bottom 96-well plates, a master plate and an induction plate. Monoclonal phages were amplified with M13 helper phage and scFv production was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG). Supernatants were then analyzed in ELISA. Furthermore, the scFv expression levels were analysed by dot blotting. The cDNA encoding the scFvs of interest were sequenced with primer PelBback (AGCCGCTGGATTGTTATTAC) at Microsynth Services. ScFv fragments were C-terminally tagged with a 6xHis-tag and vsv-G-tag by cloning the cDNA in the pUC119 vector via compatible NcoI and NotI digestion. ScFv production was induced with addition of IPTG to the culture, which was then grown for 3 hours at 30°C and 250 rpm. Then, the Escherichia coli (E.coli) periplasmic fraction was isolated by sucrose extraction and, if necessary, dialysed against PBS with three buffer changes at 4°C.

Target identification by immunoprecipitation and mass spectrometry

All antibodies for the immunoprecipitations (IPs) were produced freshly from bacterial culture. Proteins were precipitated from mixtures of two plaque and two corresponding control secretomes, using Dynabeads for His-tagged proteins. 1 ml dialysed, scFv-containing periplasmic fraction was added to the beads and incubated for 15 minutes rotating at RT. The beads were magnetically

harvested and washed. Subsequently, secretomes containing 0.14 mg of protein were added to the beads and incubated overnight at 4°C. The next day, the beads were collected from the unbound secretome fraction by using a magnet. The beads were washed and the bound proteins were eluted by addition of 200µl 100 mM glycine pH 2 and incubation of 15 minutes at RT with regular vortexing. The eluted proteins were separated from the beads magnetically and transferred to a clean tube containing 40 µl of 2 M Tris-HCl pH 8.0. The elution step was repeated and the eluted fractions combined. The precipitated fractions were stored at -20°C until further analysis. For information on unspecifically bound proteins, two control experiments were done. The precipitation was once done with magnetic Dynabeads without an antibody and once using a scFv antibody against an un-related protein, namely TWIST1. TWIST1 acts as a transcriptional regulator and inhibits myogenesis and has no known connection to atherosclerosis or heart disease.

All mass spectrometry (MS) experiments were done at the Functional Genomics Center Zurich (FGCZ), a service centre of the University of Zürich. The samples were either subjected to one-dimensional SDS-PAGE, reduction, alkylation and trypsin digest before measurement, or they were pre-treated and digested in solution. Samples were analyzed on an LTQ-Orbitrap XL mass spectrometer (Thermo Fischer Scientific), coupled to an Eksigent-Nano-HPLC system (Eksigent Technologies). Full-scan MS spectra (300-2'000 *m/z*) were acquired with a resolution of 60'000 at 400 *m/z* after accumulation to a target value of 500'000. The raw files from the mass spectrometer were converted into Mascot generic files with Mascot Distiller and searched against a human-contaminant database (human database including usual protein contaminants) using Mascot Server 2.2.

Immunoblotting and immunohistochemistry

Prior to SDS-PAGE and immunoblotting, albumin and immunoglobulins were removed from plasma samples with depletion columns (Qproteome columns, Invitrogen). Thrombi were cut in smaller pieces and were directly dissolved in SDS-PAGE loading buffer containing SDS and DTT, and heated for 5 minutes at 95°C. Proteins were separated on polyacrylamide gels by SDS-PAGE and transferred to nitrocellulose by semi-dry blotting. Membranes were then blocked with 5% non-fat dried milk powder in PBS containing 0.05% Tween-20 (MPBST). JUP was detected with scFv antibody 25G5 via the VSV-G-tag, or with anti-JUP monoclonal antibody (mAb) 2G9 (Lifespan Biosciences). Anti-VSV-G mAb was purchased from ModiQuest and horseradish peroxidase (HRP)-labelled rabbit anti-mouse antibody from Dako.

For immunohistochemical detection of JUP, tissue sections from coronary thrombi of different patients were incubated with the anti-JUP mAb 2C9. Bound antibodies were detected with HRP-labelled rabbit anti-mouse antibodies and diaminobenzidine substrate. To reduce background, tissue sections were pre-treated with 10 mM citrate buffer, pH 6.0 Eosin-hematoxylin as well as Masson-Goldner's trichrome stain were performed in all cases for general orientation and identification of tissues and structures.

	Group		
	Control (n = 13)	CAD (n = 15)	ACS (n = 11)
Age (y)	59.1 +/- 1.2	59.9 +/- 1.0	59.2 +/- 1.5
BMI (kg/m2)	27.1 +/- 1.2	28.2 +/- 1.1	25.3 +/- 2.9
BP syst. (mmHg)	132 +/- 3	133 +/- 4	134 +/- 7
BP diast. (mmHg)	80 +/- 3	79 +/- 2	84 +/- 5
Total cholesterol (mmol/l)	4.33 +/-0.35	4.32 +/- 0.22	5.02 +/- 0.33
LDL (mmol/l)	2.55 +/-0.31	2.54 +/- 0.20	3.21 +/- 0.56
HDL (mmol/l)	1.30 +/- 0.10	1.16 +/- 0.06	1.14 +/- 0.07
Triglycerides (mmol/l)	1.07 +/- 0.17	1.36 +/- 0.17	1.47 +/- 0.33
CRP	1.33 +/- 0.29 #	2.45 +/- 0.92 #	9.45 +/- 3.09 • *
Creatinine	85 +/- 3	93 +/- 4	96 +/- 10

# $p < 0.05$ compared to ACS group

• $p < 0.05$ compared to control group

* $p < 0.05$ compared to CAD group

Table 1. Patient characteristics

Claims

1. A method for diagnosis of cardiovascular disease in a patient, comprising detecting, in a sample obtained from said patient, an indicator protein selected from the group comprised of Junction plakoglobin (SEQ ID 001)), a polypeptide (SEQ ID 002) encoded by SEQ ID 004 (cDNAFLJ60), JUP peptide 1 (SEQ ID 007), JUP peptide 2 (SEQ ID 008), JUP peptide 3 (SEQ ID 009), JUP peptide 4 (SEQ ID 010), JUP peptide 5 (SEQ ID 011), JUP peptide 6 (SEQ ID 012) and JUP peptide 7 (SEQ ID 013).
2. The method of claim 1, comprising the steps of:
   - contacting, in a first contacting step, said sample with a first ligand specifically reactive to said indicator protein;
   - determining, in a quantification step, the amount of said indicator protein bound to said ligand.
3. The method according to claim 2, further comprising comparing said amount to a standard.
4. A method according to claim 2 or 3, wherein said first ligand has a fluorescent or luminescent quality and said determining is performed by fluorescence-activated flow cytometry.
5. A method according to at least one of the above claims 2 to 4, wherein the ligand is attached to a surface.
6. A method according to at least one of the above claims 2 to 5, characterized in that the quantification step comprises a second contacting step, in which the quantification step comprises the steps of contacting, in a second contacting step, said sample with a second ligand specifically reactive to said indicator protein and determining the amount of second ligand bound to said indicator protein.
7. A method according to claim 6, characterized in that said second ligand is attached or specifically attachable to an enzyme activity or an optically detectable label, particularly a peroxidase or a phosphatase activity, a luminescent activity or a fluorescence label.
8. A method according to claim 4 and 7, whereby the surface is the surface of an ELISA plate well, the sample is contacted with said surface comprising said first ligand in said first contacting step; said second ligand is contacted with said surface in said second contacting step, said sample is removed from said surface and the amount of said second ligand is determined.
9. A surface comprising a ligand specifically reactive to an indicator protein selected from the group comprised of Junction plakoglobin (SEQ ID 001)), a polypeptide (SEQ ID 002) encoded by SEQ ID 004 (cDNAFLJ60), JUP peptide 1 (SEQ ID 007), JUP peptide 2 (SEQ

ID 008), JUP peptide 3 (SEQ ID 009), JUP peptide 4 (SEQ ID 010), JUP peptide 5 (SEQ ID 011), JUP peptide 6 (SEQ ID 012) and JUP peptide 7 (SEQ ID 013)

10. A device for diagnosis of cardiovascular disease comprising a ligand specifically reactive to an indicator protein selected from the group comprised of Junction plakoglobin (SEQ ID 001), a polypeptide (SEQ ID 002) encoded by SEQ ID 004 (cDNAFLJ60), JUP peptide 1 (SEQ ID 007), JUP peptide 2 (SEQ ID 008), JUP peptide 3 (SEQ ID 009), JUP peptide 4 (SEQ ID 010), JUP peptide 5 (SEQ ID 011), JUP peptide 6 (SEQ ID 012) and JUP peptide 7 (SEQ ID 013).
11. A device according to claim 10, further comprising a surface onto which said ligand is attached.
12. A kit of parts for diagnosis of cardiovascular disease in a patient, comprising a first ligand and a second ligand, both ligands being specifically reactive to an indicator protein selected from the group comprised of Junction plakoglobin (SEQ ID 001)), a polypeptide (SEQ ID 002) encoded by SEQ ID 004 (cDNAFLJ60), JUP peptide 1 (SEQ ID 007), JUP peptide 2 (SEQ ID 008), JUP peptide 3 (SEQ ID 009), JUP peptide 4 (SEQ ID 010), JUP peptide 5 (SEQ ID 011), JUP peptide 6 (SEQ ID 012) and JUP peptide 7 (SEQ ID 013).
13. A kit of parts according to claim 12, wherein one of the first and second ligand is attached to a surface, and the other of the first and second ligand comprises an enzymatic activity or a luminescent activity.
14. The use of a surface or a device according to one of claims 9 to 11 in a method according to one of claims 1 to 8.

Fig. 1

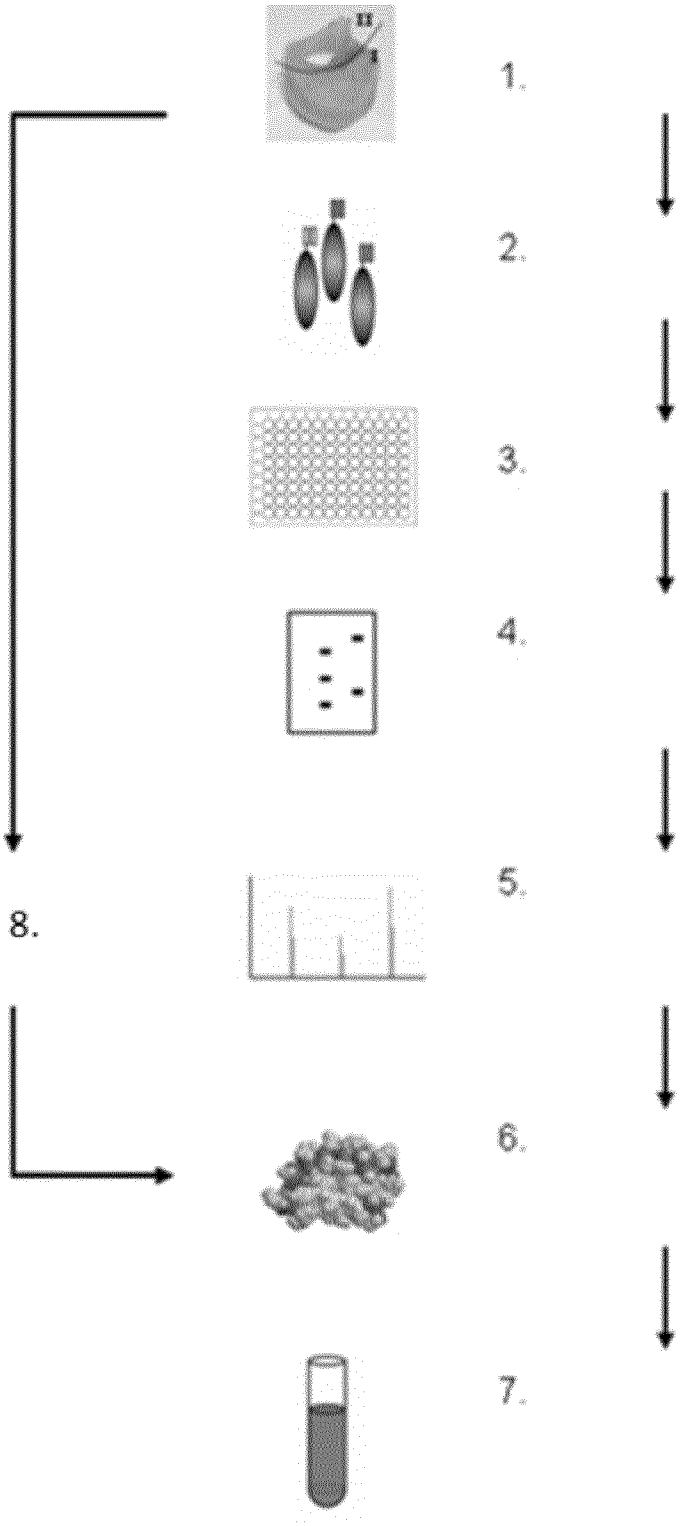
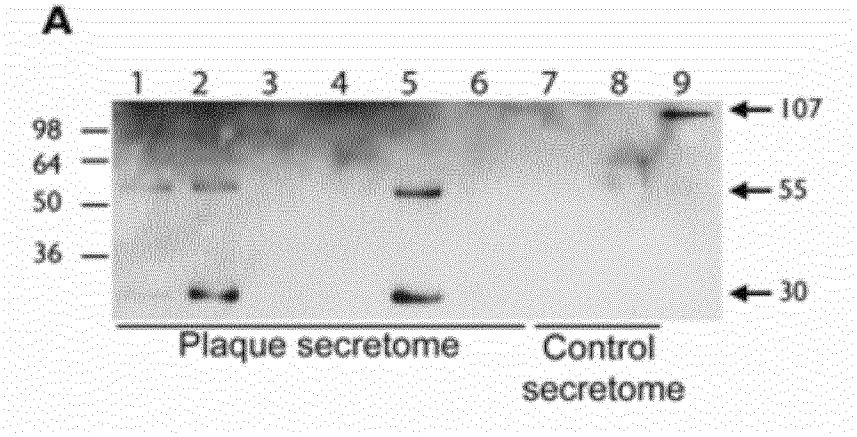


Fig. 2


A
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98
64
50
36
107
55
30
Plaque secretome
Control secretome

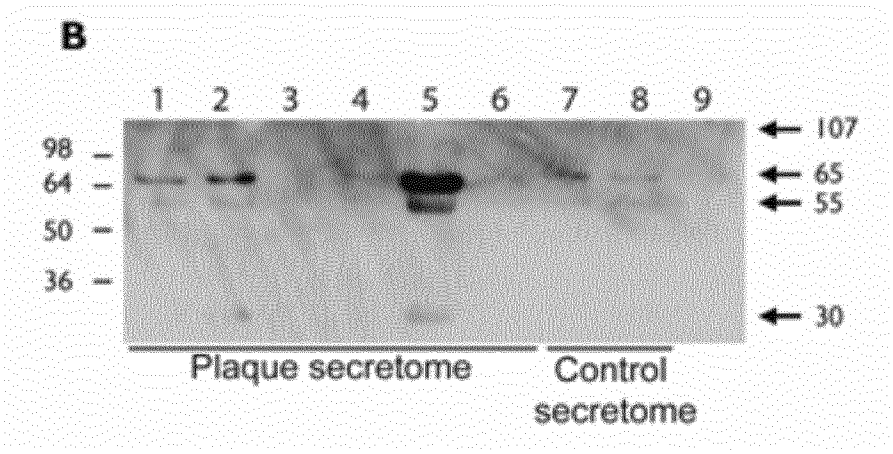
B
1 2 3 4 5 6 7 8 9
98
64
50
36
107
65
55
30
Plaque secretome
Control secretome

Fig. 3

Junction plakoglobin (81 kDa)

MEVMNLMEQPIKVTEWQQTYTYDSGIHSGANTCVPSVSSKGIMEEDEACGRQYTLKKTTTYTQGVPPSQGDL
EYQMSTTARAKRVREAMCPGVSGEDSSLLLATQVEGQATNLQRLAEPSQLLKSAIVHLINYQDDAELATRAL
PELTKLLNDEDPVVVTKAAMIVNQLSKKEASRRALMGSPQLVAAVVRTMQNTSDLDTARCTTSILHNLSHHR
EGLLAIFKSGGIPALVRMLSSPVESVLFYAITTLHNLLLYQEGAKMAVRLADGLQKMVPLLNKNNPKFLAIT
TDCLQLLAYGNQESKLIILANGGPQALVQIMRNYSYEKLLWTTSRVLKVLSVCPSNKPAIVEAGGMQALGKH
LTSNSPRLVQNCLWTLRNLSDVATKQEGLESVLKILVNQLSVDDVNVLTCATGTLSNLTCNNSKNKTLVTQN
SGVEALIHAILRAGDKDDITEPAVCALRHLTSRHPEAEMAQNSVR*LNYGIPAIVK*LLNQPNQWPLVKATIGL
IRNLALCPANHAPLQEAAVIPR*LVQLLV*KAHQDAQRHVAAGTQQPYTDGVRMEEIVEGCTGALHILARDPMN
RMEIFRLNTIPLFVQLLYSSVENIQRVAAGVLCELAQDKEAADAIDAEGASAPLMELLHSRNEGTATYAAAV
LFRISEDKNPDYRKRVSVELTNSLFKHDPAAWEAAQSMIPINEPYGDDMDATYRPMYSSDVPLDPLEMHMDM
DGDYPIDTYSDGLRPPYPTADHMLA

Junction plakoglobin variant (63 kDa)

MEVMNLMEQPIKVTEWQQTYTYDSGIHSGANTCVPSVSSKGIMEEDEACGRQYTLKKTTTYTQGVPPSQGDL
EYQMSTTARAKRVREAMCPGVSGEDSSLLLATQVEGQATNLQRLAEPSQLLKSAIVHLINYQDDAELATRAL
PELTKLLNDEDPVVVTKAAMIVNQLSKKEASRRALMGSPQLVAAVVRTMQNTSDLDTARCTTSILHNLSHHR
EGLLAIFKSGGIPALVRMLSSPVESVLFYAITTLHNLLLYQEGAKMAVRLADGLQKMVPLLNKNNPKFLAIT
TDCLQLLAYGNQESKILGATIENSRIVLQIDNAR*LAADDFRTKFETEQALR*MSVEADINGLR*RVLDELTLAR*
TDLEMQIEGLKEELAYLKKNHEEEISTLRGQVGGQVSVEVDSAPGTDLAKILSDMRSQYEVMAEQNRKDAEA
WFTSRTEELNREVAGHTEQLQMSRSEVTDLRRTLQGLEIELQSQLSMKAALEDTLAETEARFGAQLAHIQAL
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Fig. 4

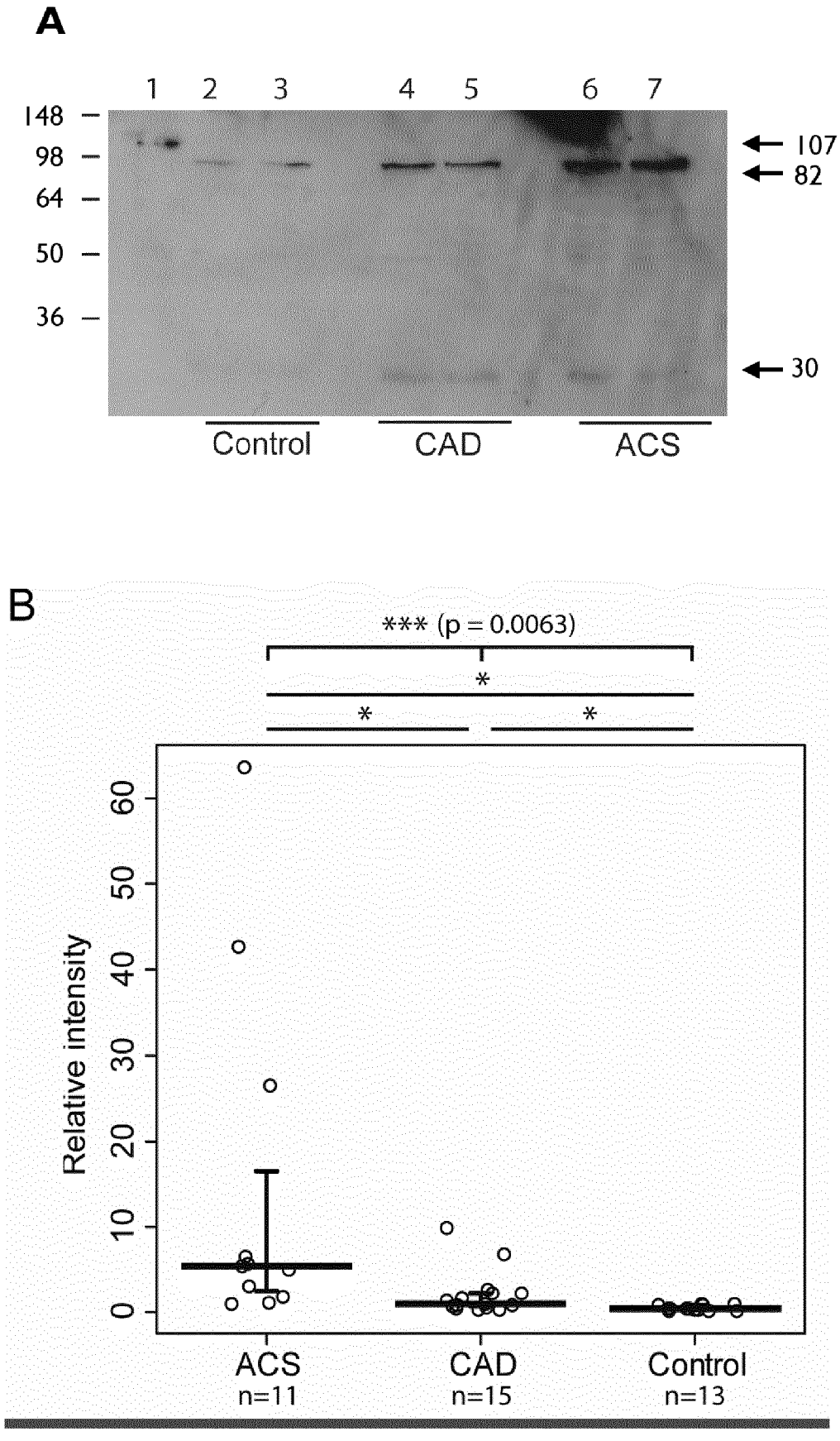
A
1 2 3 4 5 6 7
148
98
64
50
36
107
82
30
Control
CAD
ACS
B
*** (p = 0.0063)
*
*
*
Relative intensity
0
10
20
30
40
50
60
ACS
n=11
CAD
n=15
Control
n=13

Fig. 5

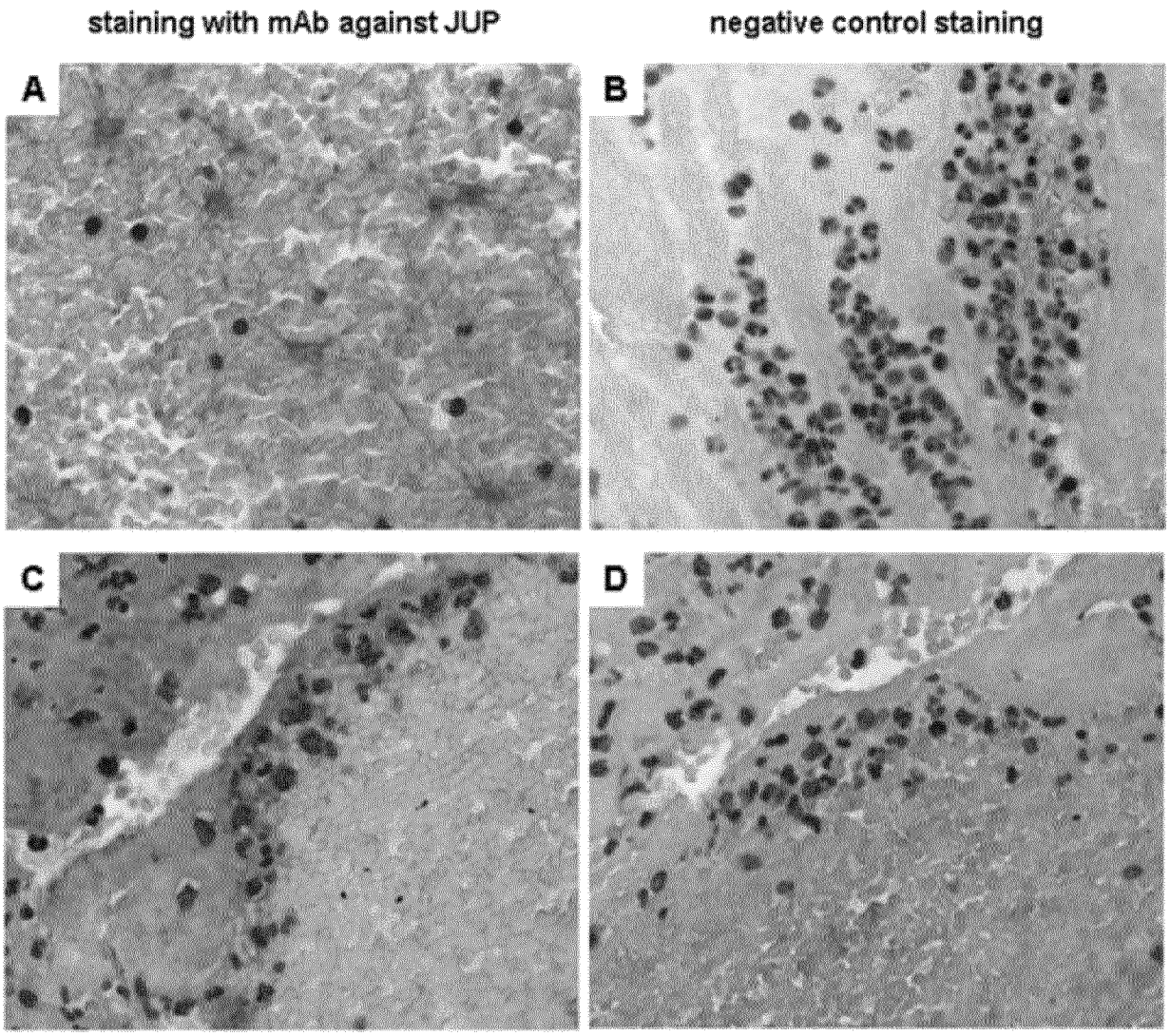
staining with mAb against JUP
negative control staining
A
B
C
D

Fig. 6

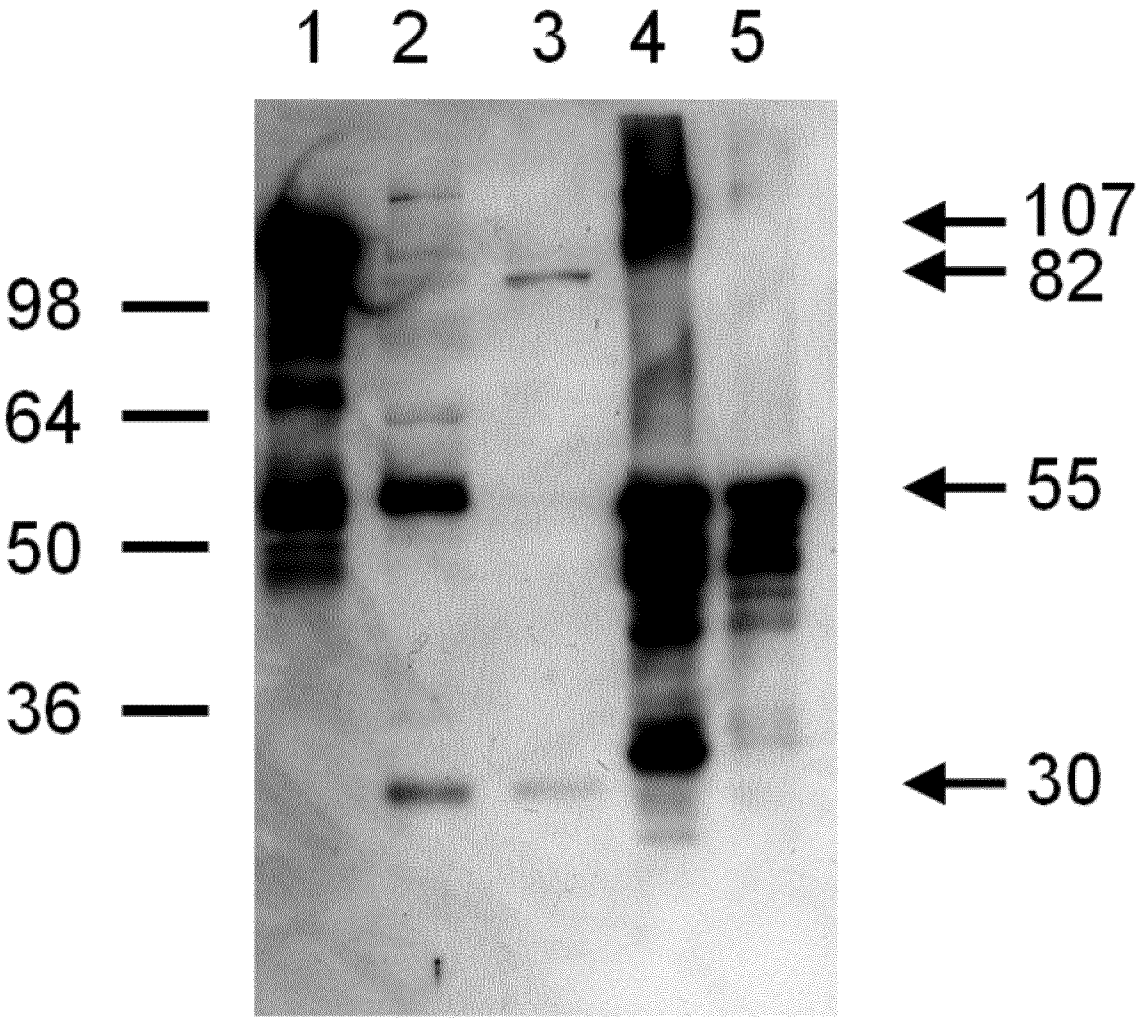

Fig. 7

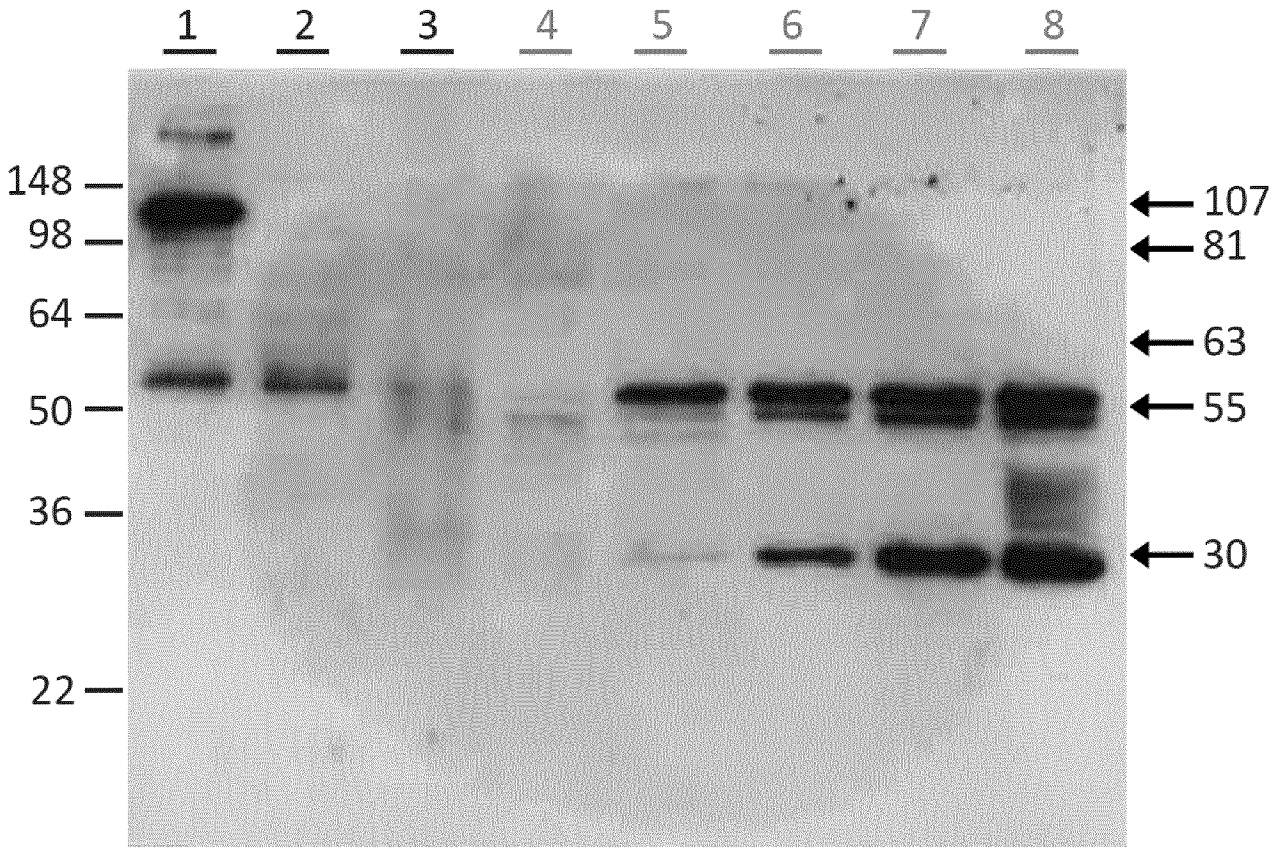

Fig 8

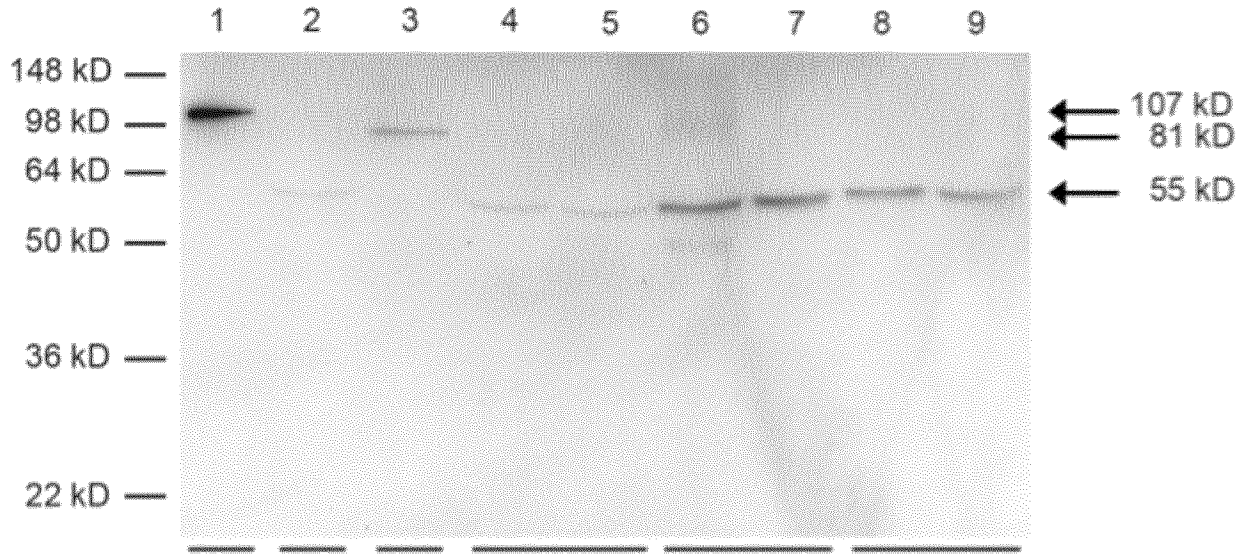

Fig. 9