(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
14 February 2019 (14.02.2019)

WIPO | PCT

(10) International Publication Number
WO 2019/028507 A1

(51) International Patent Classification:
G01N 33/68 (2006.01) *G01N 33/50* (2006.01)
G01N 33/48 (2006.01)

(21) International Application Number:
PCT/AU2018/050827

(22) International Filing Date:
08 August 2018 (08.08.2018)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
2017903138 08 August 2017 (08.08.2017) AU

(71) Applicant: **QUEENSLAND UNIVERSITY OF TECHNOLOGY** [AU/AU]; GPO Box 2434, Brisbane, Queensland 4001 (AU).

(72) Inventors: **PUNYADEERA, Chamindie**; GPO Box 2434, Brisbane, Queensland 4001 (AU). **XHANG, Xi**; GPO Box 2434, Brisbane, Queensland 4001 (AU). **SCHULZ, Benjamin**; GPO Box 2434, Brisbane, Queensland 4001 (AU).

(74) Agent: **SPRUSON & FERGUSON**; Level 6, 175 Eagle Street, Brisbane, Queensland 4000 (AU).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*

WO 2019/028507 A1

(54) Title: METHODS FOR DIAGNOSIS OF EARLY STAGE HEART FAILURE

(57) Abstract: The invention relates to methods for diagnosing the early stages of heart failure. The invention particularly relates to diagnosing class I and class II heart failure, based on the New York Heart Association (NYHA) classification system. The invention can also discriminate between healthy controls and heart failure patients in NYHA class III/IV.

METHODS FOR DIAGNOSIS OF EARLY STAGE HEART FAILURE

TECHNICAL FIELD

[0001] The present invention relates to methods for diagnosing the early stages of heart failure. The invention particularly relates to diagnosing class I and class II heart failure, based on the New York Heart Association (NYHA) classification system. The invention can also discriminate between healthy controls and heart failure patients in NYHA class III/IV.

BACKGROUND ART

[0002] Heart failure occurs when the heart muscle is weakened such that it can no longer pump sufficient blood to meet a body's requirements for blood and oxygen. In other words, the heart cannot keep up with its workload. There are a number of compensation mechanisms that come into play during the early stages of heart failure, including enlargement, increase in muscle mass, and faster pumping. Without treatment and/or lifestyle changes, eventually the compensation mechanisms are no longer effective, and the person starts to experience symptoms of heart failure, such as fatigue and breathing problems.

[0003] In the early part of the 20th century, there was no way to take measurements of cardiac function and therefore there was no consistency of diagnosis. The NYHA developed a classification system that is still used today in clinical descriptions of heart failure (The Criteria Committee of the New York Heart Association, 1994). According to the NYHA classification system, patients are placed in one of four categories, based on their limitations during physical activity, any limitations or symptoms during normal breathing and shortness of breath and/or angina.

[0004] The classification system is set out in Table 1.

[0005] Table 1. NYHA functional classification of heart failure

NYHA Class	Symptoms
I	No symptoms and no limitation during ordinary physical activity, for example, no shortness of breath or angina when walking or climbing stairs
II	Mild symptoms, for example, mild shortness of breath and/or angina, and slight limitation during ordinary physical activity
III	Marked limitation in activity due to symptoms even when walking short distances (20 – 100 metres), comfortable only at rest
IV	Severe limitations, symptoms even when at rest, any physical activity increases discomfort

[0006] Heart failure imposes substantial social and economic burdens on society, predominantly due to its high global prevalence. For example, it is estimated that 23 million people worldwide are diagnosed annually (Australian Institute of Health and Welfare (AIHW) 2011). Survival rates are also low, with about 30 % of all deaths in Australia attributed to heart failure (Palazzuoli *et al.*, 2007). The major risk factors for heart failure include age, lack of physical activity, poor eating habits leading to obesity, smoking and excessive alcohol intake (Palazzuoli *et al.*, 2007). With many countries experiencing aging populations, heart failure is expected to become an even more prevalent problem (Marian and Nambi, 2004).

[0007] There is currently no standard for heart failure diagnosis, due to the complexity of the disease. In particular, there is no simple diagnostic test for heart failure. Early changes in the structure or function of the heart such as the compensation mechanisms mentioned above, can be detected using medical imaging technology, however, it is not practical or cost-effective to be performing imaging on all potential heart failure patients.

[0008] There are a number of non-invasive risk scoring systems which were designed to assess an individual's chances of developing cardiovascular disease, such as coronary heart disease, heart failure, cardiomyopathy, congenital heart disease, peripheral vascular disease and stroke. For example, the Framingham Risk Score is an algorithm for estimating the risk over 10 years of developing coronary heart disease, peripheral artery disease and heart failure (McKee *et al.*, 1971). Other examples are the Boston Criteria for diagnosing heart failure (Carlson *et al.*, 1985), which has been shown to have the highest sensitivity and specificity (Shamsham and Mitchell, 2000) and the Duke Criteria (Harlan *et al.*, 1977). These types of criteria use a combination of patient medical history, physical examinations, routine clinical procedures and laboratory tests to reach a diagnostic conclusion (Krum *et al.*, 2006) and are particularly useful in

diagnosing advanced or severe heart failure. However, preventing progress of heart failure and clinical deterioration requires early diagnosis. An improvement in accuracy of non-invasive diagnosis of the early stages of heart failure is therefore required.

[0009] It will be clearly understood that, if a prior art publication is referred to herein, this reference does not constitute an admission that the publication forms part of the common general knowledge in the art in Australia or in any other country.

SUMMARY OF INVENTION

[0010] The present invention is broadly directed to methods for the diagnosis of early stages of heart failure, in particular, classes I and II according to the NYHA classification. In particular, the invention relates to the identification and use of biomarkers with high correlation to early stage heart failure.

[0011] In a first aspect, the present invention provides a method for detecting early stage heart failure in a subject, the method comprising analysing a biological sample obtained from the subject and determining the concentration of at least one biomarker in the sample and assigning a heart failure classification to the subject if the concentration of the at least one biomarker is either higher or lower than a predefined reference concentration of the at least one biomarker. The predefined reference concentration of the at least one biomarker can be determined from a biological sample taken from a healthy subject.

[0012] In a second aspect, the invention provides a method of detecting early stage heart failure in a subject, the method comprising analysing a biological sample obtained from the subject and determining the concentration of at least one biomarker in the sample, determining the concentration of the at least one biomarker in a biological sample obtained from a healthy subject, and assigning a heart failure classification to the subject if the concentration of the at least one biomarker in the sample from the subject is either higher or lower than the concentration of the at least one biomarker in the biological sample obtained from the healthy subject.

[0013] In a third aspect, the invention provides a method for detecting early stage heart failure in a subject, the method comprising analysing a biological sample obtained from the subject and determining the concentration of at least one biomarker in the sample, wherein the at least one biomarker is selected from the group consisting of KLK1, TCPD, S10A7, DLDH, IGHA2, CAMP, KV110, NAMPT, COPB, SPR2A and HV311, and assigning a heart failure classification to the subject if the concentration of the at least one biomarker is either higher or

lower than a predefined reference concentration of the at least one biomarker.

[0014] In a fourth aspect, the invention provides a method of detecting early stage heart failure in a subject, the method comprising analysing a biological sample obtained from the subject and determining the concentration of at least one biomarker in the sample, wherein the at least one biomarker is selected from the group consisting of KLK1, TCPD, S10A7, DLDH, IGHA2, CAMP, KV110, NAMPT, COPB, SPR2A and HV311, determining the concentration of the at least one biomarker in a biological sample obtained from a healthy subject, and assigning a heart failure classification to the subject if the concentration of the at least one biomarker in the sample from the subject is either higher or lower than the concentration of the at least one biomarker in the biological sample obtained from the healthy subject.

[0015] In a fifth aspect, the invention provides a method of screening for early stage heart failure in a subject, the method comprising analysing a biological sample obtained from the subject and determining the concentration of at least one biomarker in the sample and assigning a heart failure classification to the subject if the concentration of the at least one biomarker is either higher or lower than a predefined reference concentration of the at least one biomarker.

[0016] In a sixth aspect, the invention provides a kit for detecting the presence of at least one biomarker associated with early stage heart failure, the kit comprising a solid support having immobilized thereon at least one molecule that specifically binds to the at least one biomarker.

[0017] In a seventh aspect, the invention provides a kit for detecting the presence of at least one biomarker associated with early stage heart failure, wherein the at least one biomarker is selected from the group consisting of KLK1, TCPD, S10A7, DLDH, IGHA2, CAMP, KV110, NAMPT, COPB, SPR2A and HV311, the kit comprising a solid support having immobilized thereon at least one molecule that specifically binds to the at least one biomarker.

[0018] Throughout this specification, unless the context requires otherwise, the words "comprise", "comprises" and "comprising" will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

[0019] Any of the features described herein can be combined in any combination with any one or more of the other features described herein within the scope of the invention.

BRIEF DESCRIPTION OF DRAWINGS

[0020] Figure 1 is a graph showing the abundance of peptides from each protein identified by ProteinPilot database searches (Table 3) as determined from extracted ion chromatograms of

LC-ESI-MS/MS data.

[0021] Figure 2 is a series of graphs comparing the relative abundance of various salivary proteins in healthy controls and heart failure patients in NYHA Class I and Class III/IV, as determined by SWATH-MS. Figure 2A, individual proteins validated by SWATH-MS; Figure 2B, SPLC2 (BNP:Control); Figure 2C, KLK1 (BNP:Control); Figure 2D, KLK1:SPLC2 (BNP:Control); Figure 2E, S10A7 (BNP:Control); Figure 2F, S10A7:SPLC2 (BNP:Control); Figure 2G, AACT (BNP:Control); and Figure 2H, AACT:SPLC2 (BNP:Control).

[0022] Figure 3 is a series of dot plots comparing the ratio of select salivary proteins in healthy controls and heart failure patients. Figure 3A, KLK1:SPLC2; Figure 3B, S10A7:SPLC2; and Figure 3C, AACT:SPLC2.

[0023] Figures 4A, 4B and 4C are ROC curves for the salivary protein ratios in Figure 3. Figure 4A, KLK1:SPLC2; Figure 4B, S10A7:SPCL2; and Figure 4C, AACT:SPLC2.

[0024] Figure 5 is a series of graphs comparing the relative abundance of various salivary proteins (KV110, NAMPT, COPB, SPR2A and HV311) in healthy controls and heart failure patients in NYHA Class I and Class III/IV, as determined by SWATH-MS.

[0025] Figure 6 is an overlay of ROC curves for comparisons of the combination of salivary proteins shown in Figure 5 between various cohorts (NYHA Class I, NYHA Class III/IV and controls).

[0026] Figure 7 is a series of graphs comparing the relative abundance of various salivary proteins (KLK1, TCPD, S10A7, DLDH, IGHA2 and CAMP) in healthy controls and heart failure patients in NYHA Class I and Class III/IV, as determined by SWATH-MS.

[0027] Figure 8 is an overlay of ROC curves for comparisons of the combination of salivary proteins shown in Figure 7 between various cohorts (NYHA Class I, NYHA Class III/IV and controls).

[0028] Figure 9 is a series of graphs comparing the concentration of various salivary proteins (S10A7, KLK1 and CAMP) in healthy controls, individuals with high risk of developing heart failure and heart failure patients, as determined by immunoassays; and ROC curves for comparisons of the combination salivary proteins. A prediction score was generated by combining the concentration of these salivary proteins. Figure 9A, S10A7; Figure 9B, CAMP; Figure 9C, KLK1; Figure 9D, combined prediction score of the salivary proteins; Figure 9E,

ROC curve for comparison of the combination of salivary proteins between heart failure patients and controls; Figure 9F, ROC curve for comparison of the combination of salivary proteins between SCREEN-HF cohorts and controls.

[0029] Figure 10 is a graph showing the prediction score between study subjects who have developed cardiovascular disease after enrolment in the study, and those who have no cardiovascular disease-related hospital admission.

[0030] Figure 11 (A) Western blotting of KLK1, TCPD, S10A7, DLDH, IGHA2 and CAMP in saliva samples of 6 healthy controls and 6 heart failure patients. (B) Average relative band intensity with standard error of KLK1, TCPD, S10A7, DLDH, IGHA2 and CAMP in saliva samples of healthy control and heart failure patients.

[0031] Figure 12 is a Western blot of S10A7 in additional saliva samples of 12 healthy controls and 12 heart failure patients.

DESCRIPTION OF EMBODIMENTS

Abbreviations

[0032] The following abbreviations are used throughout:

AACT = alpha 1 anti-chymotrypsin

BNP = brain natriuretic peptide

CAMP = Cathelicidin antimicrobial peptide

COPB = coatomer subunit beta

DLDH = Dihydrolipoyl dehydrogenase, mitochondrial

ESI = electron spray ionization

GELS = gelsolin

h = hour

HV311 = Ig heavy chain V-III region KOL

IGHA2 = Ig alpha-2 chain C region

IGJ = immunoglobulin J chain

IQR = interquartile range

KLK1 = kallikrein 1

KV110 = Ig kappa chain V-I region HK102

LC = liquid chromatography

LC-ESI-MS/MS = liquid chromatograph-electrospray ionization-tandem mass spectrometry

LPLC1 = long palate lung and nasal epithelium carcinoma-associated protein 1

min = minute(s)

MMP9 = matrix metalloproteinase-9

MS = mass spectrometry

MS/MS = tandem mass spectrometry

NAMPT = nicotinamide phosphoribosyltransferase

NPV = negative predictive value

NYHA = New York Heart Association

PBS = phosphate buffered saline

PPV = positive predictive value

rcf = relative centrifugal force

ROC = receiver operating characteristic

s = second(s)

S10A7 = S100 calcium binding protein A7

SPLC2 = short palate lung and nasal associated protein 2

SPR2A = small proline-rich protein 2A

SWATH = sequential window acquisition of all theoretical fragment ion spectra

TCPD = T-complex protein 1 subunit delta

TOF = time of flight

VIME = vimentin

[0033] The present invention is predicated in part on the discovery that proteins in a biological sample taken from a subject with early stage heart failure are differentially abundant when compared to a biological sample taken from a healthy subject. The present inventors have used high abundant protein depletion and SWATH-MS to identify salivary proteins as putative biomarkers having diagnostic utility in the early stages of heart failure.

[0034] Accordingly, in a first aspect, the invention provides a method for detecting early stage heart failure in a subject, the method comprising analysing a biological sample obtained from the subject and determining the concentration of at least one biomarker in the sample and assigning a heart failure classification to the subject if the concentration of the at least one biomarker is either higher or lower than a predefined reference concentration of the at least one biomarker. The predefined reference concentration of the at least one biomarker can be determined from a biological sample taken from a healthy subject.

[0035] For the purposes of this invention, the phrase "early stage(s)" to describe a stage of heart failure, refers to the functional classifications NYHA Class I and/or NYHA Class II, as defined by the New York Heart Association.

[0036] The term "biological sample" is used herein to refer to a sample that is extracted from a subject. The term encompasses untreated, treated, diluted or concentrated biological samples. The biological sample obtained from the subject can be any suitable sample, such as whole blood, serum or plasma. Preferably, the biological sample is obtained from the buccal cavity of the subject. The biological sample can therefore be sputum or saliva. In accordance with the invention providing a non-invasive, cost-effective method for diagnosing early stage heart failure, the biological sample obtained from the subject is preferably saliva.

[0037] The at least one biomarker is a protein present in the biological sample that has been identified as having a correlation with early stage heart failure. The biological sample can be analysed for the concentration of at least one, two, three, four, five, six, *etc.,* biomarkers. For example, the at least one biomarker can be any number of proteins selected from the group

consisting of KLK1, TCPD, S10A7, DLDH, IGHA2, CAMP, KV110, NAMPT, COPB, SPR2A and HV311. In one embodiment, the at least one biomarker is selected from the group of proteins consisting of KLK1, TCPD, S10A7, DLDH, IGHA2 and CAMP. Preferably, the at least one biomarker is a biomarker panel consisting of two, three, four, five, or all six of these proteins. In a particularly preferred embodiment, the biomarker panel comprises KLK1, S10A7, and CAMP. In an alternative embodiment, the at least one biomarker is selected from the group consisting of KV110, NAMPT, COPB, SPR2A and HV311. In a particularly preferred embodiment, the at least one biomarker is a biomarker panel consisting of two, three, four or all five of these proteins.

[0038] The predefined reference concentration for a biomarker can be in the form of a range of concentrations, such that a concentration of a biomarker outside the range is indicative of an early stage of heart failure. Alternatively, the predefined reference concentration for a biomarker can be in the form of a particular value, such that a concentration of a biomarker either higher or lower than the value is indicative of an early stage of heart failure. Therefore, for each biomarker used in the detection of early stage heart failure in a subject, a predefined reference concentration of the biomarker in a biological sample from a healthy subject has been determined, or is known.

[0039] In the context of this invention and with respect to determining predefined reference concentrations of the at least one biomarker from a biological sample taken from a healthy subject, a "healthy subject" is a subject that does not have heart failure. That is, a healthy subject is a subject that is not suffering any outward symptoms of heart failure, and would not be classified in NYHA Class I or Class II.

[0040] The present inventors have surprisingly found that particular proteins have increased abundance in saliva from subjects classified in NYHA Class I or Class II when compared to the abundance of the same protein in a healthy subject. Conversely, particular proteins have decreased abundance in saliva from subjects classified in NYHA Class I or Class II when compared to the abundance of the same protein in a healthy subject.

[0041] Although a heart failure classification can be assigned to a subject based on the concentration of just one biomarker in a biological sample from the subject, it is advantageous to base the assignation of classification on the concentration of two, three, four, five or more biomarkers in the biological sample, as a higher degree of certainty of classification could be achieved by using more biomarkers.

[0042] When using a biomarker panel consisting of two or more biomarkers to detect early

stage heart failure in a subject, the panel can consist of biomarkers that have a higher concentration in saliva from a heart failure subject than for the same biomarkers in saliva from a healthy subject. Alternatively, the panel can consist of biomarkers that have a lower concentration in saliva from a heart failure subject than from the same biomarkers in saliva from a healthy subject. In a further alternative, the panel can consist of a combination of biomarkers, wherein at least one biomarker has a higher concentration in saliva from a heart failure subject than for the same biomarker in saliva from a healthy subject and at least one biomarker has a lower concentration in saliva from a heart failure subject than for the same biomarker in saliva from a healthy subject.

[0043] In a second aspect, the invention provides a method of detecting early stage heart failure in a subject, the method comprising analysing a biological sample obtained from the subject and determining the concentration of at least one biomarker in the sample, determining the concentration of the at least one biomarker in a biological sample obtained from a healthy subject, and assigning a heart failure classification to the subject if the concentration of the at least one biomarker in the sample from the subject is either higher or lower than the concentration of the at least one biomarker in the biological sample obtained from the healthy subject.

[0044] The concentration of the at least one biomarker in a biological sample, whether from a potential heart failure subject or a healthy subject, can be determined by any suitable means for determining protein concentration. For example, the concentration can be determined by mass spectrometry analysis. Comparison of peak intensity for a particular biomarker in the mass spectrum of a sample from a potential heart failure subject and the mass spectrum of a sample from a healthy subject can provide an indication of the relative difference in abundance of the biomarker in the two samples. A more accurate comparison can be obtained using SWATH-MS as detailed in the Examples, below.

[0045] Alternatively, determining the concentration of a least one biomarker in a biological sample can be undertaken using a reagent or reagents that specifically bind to the at least one biomarker. For example, the reagent could comprise an antibody to an epitope of the biomarker, with the antibody optionally including a label (*e.g.* a fluorescent tag) for detecting the presence of the antibody-biomarker complex.

[0046] In a third aspect, the invention provides a method for detecting early stage heart failure in a subject, the method comprising analysing a biological sample obtained from the subject and determining the concentration of at least one biomarker in the sample, wherein the at

least one biomarker is selected from the group of proteins consisting of KLK1, TCPD, S10A7, DLDH, IGHA2, CAMP, KV110, NAMPT, COPB, SPR2A and HV311, and assigning a heart failure classification to the subject if the concentration of the at least one biomarker is higher or lower than a predefined reference concentration of the at least one biomarker. The predefined reference concentration of the at least one biomarker can be determined from a biological sample taken from a healthy subject.

[0047] The biological sample can be analysed for the concentration of at least one, two, three, four, five, six, seven, eight, nine, ten, or all eleven of the proteins. Although a heart failure classification can be assigned to a subject based on the concentration of just one protein from the biological sample, it is advantageous to base the assignation of classification on the concentration of two, three, four, five, six, seven, eight, nine, ten, or eleven proteins in the biological sample, as a higher degree of certainty of classification could be achieved by using more biomarkers.

[0048] The certainty of classification can be assessed by determining the sensitivity and specificity of the comparative data.

[0049] In a fourth aspect, the invention provides a method of detecting early stage heart failure in a subject, the method comprising analysing a biological sample obtained from the subject and determining the concentration of at least one biomarker in the sample, wherein the at least one biomarker is selected from the group of proteins consisting of KLK1, TCPD, S10A7, DLDH, IGHA2, CAMP, KV110, NAMPT, COPB, SPR2A and HV311, determining the concentration of the at least one biomarker in a biological sample obtained from a healthy subject, and assigning a heart failure classification to the subject if the concentration of the at least one biomarker in the sample from the subject is higher or lower than the concentration of the at least one biomarker in the biological sample obtained from the healthy subject.

[0050] In a fifth aspect, the invention provides a kit for detecting the presence of at least one biomarker associated with early stage heart failure, the kit comprising a solid support having immobilized thereon at least one molecule that specifically binds to the at least one biomarker.

[0051] The at least one molecule that specifically binds to the at least one biomarker can be any suitable molecule. Preferably, the at least one molecule comprises an antibody that specifically binds to the at least one biomarker. The solid support can therefore have one, two, three, four, *etc.* antibodies immobilized thereon.

[0052] The solid support can be any suitable material that can be modified as appropriate for

the immobilization of antibodies and is amenable to at least one detection method. Representative examples of materials suitable for the solid support include glass and modified or functionalized glass, plastics (including acrylics, polystyrene and copolymers of styrene and other materials, polypropylene, polyethylene, polybutylene, polyurethanes, Teflon, *etc.*), polysaccharides, nylon or nitrocellulose, resins, silica or silica-based materials including silicon and modified silicon, carbon, metals, inorganic glasses and plastics. The solid support can allow for optical detection without appreciably fluorescing.

[0053] The solid support can be planar, although other configurations of substrates can be utilized. For example, the solid support could be a tube with antibodies placed on the inside surface.

[0054] In a sixth aspect, the invention provides a kit for detecting the presence of at least one biomarker associated with early stage heart failure, wherein the at least one biomarker is selected from the group consisting of KLK1, TCPD, S10A7, DLDH, IGHA2, CAMP, KV110, NAMPT, COPB, SPR2A and HV311, the kit comprising a solid support having immobilized thereon at least one molecule that specifically binds to the at least one biomarker.

EXAMPLES

EXAMPLE 1

Materials and Methods

Study participants

[0055] This study was approved by the University of Queensland Medical Ethical Institutional Board and Mater Health Services Human Research Ethics Committee and by the Royal Brisbane and Women's Hospital Research Governance. All study participants were >18 years of age and gave informed consent before sample collection. The exclusion criteria for the healthy controls were based on a simple questionnaire asking volunteers to indicate the existence of any comorbid diseases and oral diseases (*e.g.* periodontal disease and gingivitis, autoimmune, infectious, musculoskeletal, or malignant disease, and recent operation or trauma). If any condition existed, the participants were excluded from the study. The volunteers were from Caucasian and Asian ethnic origins, had no symptoms of fever or cold, and had good oral hygiene.

[0056] A total of 30 healthy controls and 33 symptomatic heart failure patients were

recruited from the University of Queensland, the Mater Adult Hospital or the Royal Brisbane and Women's Hospital in Brisbane, Australia from January 2012 to July 2014. Patients were classified using New York Heart Association (NYHA) functional classification system by cardiologists at Mater Adult Hospital and Royal Brisbane and Women's Hospital based on their clinical symptoms. All patients participating in the study were classified as NYHA class III or IV patients. The mean age of heart failure patients was 67.6 and the mean age of healthy controls was 49.7. Males comprised 63.3% of the heart failure patient cohort and 43.3% of the healthy control cohort.

Saliva sample collection

[0057] Whole mouth unstimulated resting saliva was collected from early and late stage heart failure patients and from healthy controls according to previously published methods (Martinet W *et al.*, 2003; Punyadeera C *et al.*, 2011; Foo JY *et al.*, 2013; Castagnola M *et al.*, 2011; Helmerhorst EJ and Oppenheim FG, 2007; Loo JA *et al.*, 2010). Volunteers were asked to refrain from eating or drinking (except for water) for at least 30 minutes prior to saliva collection. Volunteers were asked to rinse their mouth with water to remove food particles and debris, to tilt their head forward and down, pool saliva in their mouth and expectorate into Falcon tubes (50 mL, Greiner, Germany) on ice. Samples were transferred to the laboratory on dry ice and aliquoted into protein lo-bind Eppendorf tubes (Eppendorf, USA) and stored at -80 °C for later analysis.

Total protein concentrations in saliva samples

[0058] For initial screening, total protein concentrations in saliva samples from patients (n=10) and controls (n=10) were measured using a 2D Quant kit (GE Healthcare Bio-Sciences AB, Sweden). The absorbance was measured at 480 nm using a SpectraMax® 190 plate reader (Molecular Devices, LLC, California, USA). Quick Start™ Bradford Protein Assay (Bio-Rad, USA) was used to quantify the total protein concentrations in saliva samples from patients (n=30) and controls (n=30) for the SWATH-MS validation (see below).

Saliva sample preparation for mass spectrometry analysis

[0059] Saliva samples normalized for protein content collected from heart failure patients and healthy controls were separately pooled. Equal amounts of total protein from each individual were pooled to give 10 mg of total pooled protein each for controls and patients. Pooled samples were processed with a ProteoMiner® small capacity kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Bead packed bed (20 uL) was added to pooled saliva and

incubated at 25 °C for 16 hours on a rotational shaker. Beads were pelleted by centrifugation at 1,000 relative centrifugal force (rcf) for 1 minute and the supernatant discarded. Beads were washed three times with phosphate buffered saline (PBS) and bound proteins were eluted in 8 M urea, 2% CHAPS and 5 % acetic acid (20 µL). Eluted proteins were precipitated by the addition of 1:1 methanol:acetone (80 µL), incubation at -20 °C for 16 hours, and centrifugation at 18,000 rcf for 10 minutes. The protein pellets were resuspended in 50 mM Tris-HCl buffer pH 8 with 1% SDS. Cysteines were reduced by addition of DTT to 10 mM and incubation at 95 °C for 10 min, and then alkylated by addition of acrylamide to 25 mM and incubation at 23 °C for 1 h. Proteins were precipitated as above, resuspended in 50 mM NH_4HCO_3 (50 µL) with proteomics grade trypsin (1 µg) (SigmaAlrdich, USA) and incubated at 37 °C for 16 h.

[0060] For SWATH-MS validation using individual samples, saliva containing 50 µg of total protein was supplemented with an equal volume of 100 mM Tris-HCl buffer pH 8, 2 % SDS and 20 mM DTT and incubated at 95 °C for 10 min. Proteins were then alkylated, precipitated and digested as above.

Mass spectrometry and data analysis

[0061] Peptides were desalted using C18 Zip Tips (Millipore, USA) and analyzed by LC-ESI-MS/MS using a Prominence nanoLC system (Shimadzu, Japan) on a Triple TOF 5600 mass spectrometer with a Nanospray III interface (AB SCIEX) essentially as previously described (Foo *et al.*, 2013; Ovchinnikov *et al.*, 2012). Approximately 2 µg of peptides were desalted on an Agilent C18 trap (300 Å pore size, 5 µm particle size, 0.3 mm i.d. x 5 mm) at a flow rate of 30 µL/min for 3 min, and then separated on a Vydac EVEREST reversed-phase C18 HPLC column (300 Å pore size, 5 µm particle size, 150 µm i.d. x 150 mm) at a flow rate of 1 µL/min. Peptides were separated with a gradient of 1-10% buffer B over 2 min followed by 10-60 % buffer B over 45 min, with buffer A (1 % acetonitrile and 0.1 % formic acid) and buffer B (80 % acetonitrile with 0.1 % formic acid). Gas and voltage settings were adjusted as required. An MS-TOF scan from an *m/z* of 350-1800 was performed for 0.5 s followed by information dependent acquisition of MS/MS with automated CE selection of the top 20 peptides from *m/z* of 40-1800 for 0.05 s per spectrum. Identical LC parameters were used for SWATH analyses, with an MS-TOF scan from an *m/z* of 350-1800 for 0.05 s followed by high sensitivity information independent acquisition with 26 *m/z* isolation windows with 1 *m/z* window overlap each for 0.1 s across an *m/z* range of 400-1250. Collision energy was automatically assigned by the Analyst software (AB SCIEX) based on *m/z* window ranges.

[0062] Proteins were identified using ProteinPilot (AB SCIEX), searching the LudwigNR database (downloaded from http://apcf.edu.au as at 27 January 2012; 16,818,973 sequences; 5,891,363,821 residues) using standard settings: Sample type, identification; Cysteine alkylation, none; Instrument, Triple-TOF 5600; Species, no restriction; ID focus, biological modifications; Enzyme, trypsin; Search effort, thorough ID. False discovery rate analysis using ProteinPilot was performed on all searches. Peptides identified with greater than 99 % confidence and with a local false discovery rate of less than 1 % were included for further analysis. Semi-quantitative comparison of protein abundance based on protein rank, score, percent peptide coverage and number of peptides was performed as previously described (Bailey and Schulz, 2013). Extracted ion chromatograms were obtained using Peak View 1.1. The ProteinPilot data were used as ion libraries for SWATH analyses. Protein abundance was measured automatically with Peak View 1.2 Software with standard settings. The abundance of each protein was normalized to the total abundance of identified proteins in each individual sample, log-transformed and compared using ANOVA. Data generated with SWATH analyses were analysed for protein significance using an open-sourced statistical package MSstats (Clough *et al.*, 2012; Chang *et al.*, 2012) based on R (R Development Core Team, 2011). Group comparison function was used to compare significant changes in protein abundance between heart failure patients and controls.

EXAMPLE 2

Identification of proteins via LC-ESI-MS/MS

[0063] Putative novel salivary protein biomarkers for heart failure were identified by separately pooling saliva from patients with elevated BNP and healthy controls, performing ProteoMiner dynamic range reduction, digesting proteins with trypsin and identifying peptides using LC-ESI-MS/MS and database searching. To detect proteins with altered abundance between heart failure patients and controls, a semi-quantitative approach was used to compare the rank, score, precent peptide coverage and number of peptides identified for each protein. This semi-quantitative approach identified multiple putative differentially abundant proteins as presented in Table 2.

Table 2. Differentially abundant salivary proteins, comparing heart failure patients to controls

Protein Accession	N			Score			%Cov			Peptides(95%)		
	B	C	²	B	C	²	B	C	²	B	C	²
sp|Q96DR5|SPLC2_HUMAN	53	16	37	4	18.51	-14.51	8.83	40.96	-32.12	2	9	-7
sp|P22079|PERL_HUMAN	87*	27	60		12	-12		13.06	-13.06		6	-6
sp|Q08380|LG3BP_HUMAN	72	25	47	2	12.02	-10.02	2.22	18.12	-15.90	1	6	-5
sp|P06396|GELS_HUMAN	15	7	8	13.44	22.07	-8.63	18.16	27.62	-9.46	7	12	-5
sp|P08670|VIME_HUMAN	79	51	28	2	8	-6	2.15	9.23	-7.08	1	4	-3
sp|P07237|PDIA1_HUMAN	61	38	23	4	10	-6	3.54	10.24	-6.70	2	5	-3
sp|P07737|PROF1_HUMAN	21	30	-9	12	11.62	0.38	55.71	55.71	0.00	7	7	0
sp|P01833|PIGR_HUMAN	18	33	-15	12	10.59	1.41	12.30	8.51	3.79	6	5	1
sp|P04075|ALDOA_HUMAN	22	52	-30	10	8	2	25.27	18.96	6.31	5	4	1
sp|P06870|KLK1_HUMAN	47	101	-54	4.09	2	2.09	14.12	9.16	4.96	3	1	2
sp|P0CG06|LAC3_HUMAN	31	74	-43	8	4	4	46.23	32.08	14.15	4	2	2
sp|P01591|IGJ_HUMAN	45	128*	-83	5.54		5.54	23.27		23.27	4		4
sp|P14780|MMP9_HUMAN	42	128*	-86	6		6	5.94		5.94	3		3
sp|Q8TDL5|LPLC1_HUMAN	10	60	-50	17.07	6	11.07	22.73	8.88	13.85	11	3	8

B, BNP; C, Control; ², BNP - Control; N, protein rank; *, not identified, lowest rank.

[0064] For initial validation of these putative biomarkers, the abundance of peptides from each protein identified by ProteinPilot database searches (Table 3) as determined from extracted ion chromatograms of LC-ESI-MS/MS data (Figure 1) was compared. Comparison of peptide abundances identified two proteins with significantly higher abundance (long palate, lung and nasal epithelium carcinoma-associated protein 1, LPLC1 (P=0.0004) and matrix metalloproteinase-9, MMP9 (P=0.02)) and two with significantly lower abundance (gelsolin, GELS (P=0.03) and short palate lung and nasal associated protein 2, SPLC2 (P=0.0003)) in heart failure patients compared with the control samples. Several additional proteins showed large changes in abundance (kallikrein 1, KLK1; immunoglobulin J chain, IGJ; and vimentin, VIME) which could not be statistically compared due to the small number of confidently identified peptides detected. This initial analysis therefore identified several putative salivary protein biomarkers of heart failure.

Table 3. Relative abundances of peptides for each protein identified using ProteinPilot

Protein Accession	Peptide	ZMass	*m/z*	*z*	Score
sp|P01591|IGJ_HUMAN	CYTAVVPLVYGGETK	0.0008	835.92	2	16
sp|P01591|IGJ_HUMAN	IIVPLNNR	-0.0028	469.78	2	8
sp|P01591|IGJ_HUMAN	MVETALTPDACYPD	0.0015	798.84	2	10
sp|P01833|PIGR_HUMAN	CPLLVDSEGWVK	-0.0043	708.85	2	10
sp|P01833|PIGR_HUMAN	DGSFSVVITGLR	-0.0022	625.83	2	15
sp|P01833|PIGR_HUMAN	ILLNPQDK	-0.0031	470.77	2	8
sp|P01833|PIGR_HUMAN	LVSLTLNLVTR	-0.0015	614.88	2	16
sp|P01833|PIGR_HUMAN	NADLQVLKPEPELVYEDLR	0.0104	747.73	3	18
sp|P01833|PIGR_HUMAN	VYTVDLGR	-0.0021	461.74	2	7
sp|P06396|GELS_HUMAN	AQPVQVAEGSEPDGFWEALGGK	-0.0036	1136.54	2	16
sp|P06396|GELS_HUMAN	EPAHLMSLFGGKPMIIYK	0.0006	508.77	4	10
sp|P06396|GELS_HUMAN	EVQGFESATFLGYFK	0.0017	861.92	2	9
sp|P06396|GELS_HUMAN	HVVPNEVVVQR	0.0011	638.36	2	10
sp|P06870|KLK1_HUMAN	LTEPADTITDAVK	-0.0024	687.35	2	12
sp|P06870|KLK1_HUMAN	QADEDYSHDLMLLR	-0.0019	853.39	2	12
sp|P08670|VIME_HUMAN	EEAENTLQSFR	-0.0073	662.30	2	11
sp|P08670|VIME_HUMAN	EYQDLLNVK	-0.001	561.29	2	10
sp|P08670|VIME_HUMAN	ILLAELEQLK	-0.0036	585.35	2	8
sp|P0CG06|LAC3_HUMAN	AAPSVTLFPPSSEELQANK	0.0026	662.67	3	16
sp|P0CG06|LAC3_HUMAN	AAPSVTLFPPSSEELQANK	0.0024	993.51	2	16
sp|P0CG06|LAC3_HUMAN	SYSCQVTHEGSTVEK	-0.0038	575.92	3	12
sp|P0CG06|LAC3_HUMAN	YAASSYLSLTPEQWK	0.0013	872.43	2	16
sp|P0CG06|LAC3_HUMAN	YAASSYLSLTPEQWK	0.0031	581.95	3	17
sp|P14780|MMP9_HUMAN	LGLGADVAQVTGALR	-0.0032	720.90	2	9
sp|P14780|MMP9_HUMAN	QLSLPETGELDSATLK	0.0004	851.44	2	11
sp|P14780|MMP9_HUMAN	SLGPALLLLQK	-0.0047	576.86	2	11
sp|Q8TDL5|LPLC1_HUMAN	ALGFEAAESSLTK	-0.0029	662.33	2	19
sp|Q8TDL5|LPLC1_HUMAN	DALVLTPASLWKPSSPVSQ	-0.0008	998.53	2	15
sp|Q8TDL5|LPLC1_HUMAN	GDQLILNLNNISSDR	-0.011	836.42	2	14
sp|Q8TDL5|LPLC1_HUMAN	ILTQDTPEFFIDQGHAK	0.0046	653.99	3	13
sp|Q8TDL5|LPLC1_HUMAN	IPLDMVAGFNTPLVK	-0.0016	807.94	2	19
sp|Q8TDL5|LPLC1_HUMAN	SGVPVSLVK	-0.0006	443.27	2	9
sp|Q8TDL5|LPLC1_HUMAN	SSIGLINEK	-0.0023	480.76	2	10
sp|Q96DR5|SPLC2_HUMAN	FVNSVINTLK	-0.0028	567.82	2	10
sp|Q96DR5|SPLC2_HUMAN	ISNSLILDVK	-0.0023	551.32	2	14
sp|Q96DR5|SPLC2_HUMAN	LEPVLHEGLETVDNTLK	0.0002	636.34	3	13
sp|Q96DR5|SPLC2_HUMAN	LLNNVISK	-0.0029	450.77	2	9
sp|Q96DR5|SPLC2_HUMAN	LLPTNTDIFGLK	-0.0007	666.37	2	10
sp|Q96DR5|SPLC2_HUMAN	VDLGVLQK	-0.0006	436.26	2	10

EXAMPLE 3

Validation with SWATH-MS

[0065] To validate the novel putative biomarkers identified from ProteoMiner® analysis of pooled samples, SWATH-MS detection was performed on individual saliva samples collected from heart failure patients and controls. Unbiased SWATH-MS proteomic comparison of saliva from heart failure patients and controls resulted in the identification of seven proteins with >2-

fold difference in abundance and adjusted $P<0.01$. This included the SPLC2 protein identified by ProteoMiner analysis as a putative heart failure biomarker. The relative abundance of SPLC2 was 1.89-fold lower in heart failure patients than in controls. Saliva with high specificity (almost complete group separation) (see Figure 2A, adjusted P<0.0001), validated SPLC2 as a salivary protein biomarker for heart failure. KLK1 was also putatively identified by ProteoMiner analysis as a potential biomarker due to its higher abundance in saliva from heart failure patients then in saliva from controls (Figure 1). The increased abundance of KLK1 was also validated by SWATH-MS analysis, which showed a 1.3-fold increase in abundance in heart failure patients compared to controls (Figure 2B, adjusted P=<0.0001).

[0066] As SPLC2 abundance was decreased and KLK1 abundance increased in heart failure patients compared to controls, the utility of a ratio of the abundance of these individually validated biomarkers for identifying heart failure was investigated. A large and highly significant discrimination between heart failure patients and controls was observed, with a 5.3-fold difference in ratio and high specificity (Figure 2C, P=0.00001). A Receiver Operating Characteristic (ROC) curve analysis was undertaken to determine the diagnostic power of SPLC2 and KLK1 as biomarkers. The analysis of KLK:SPLC2 (Figure 3A, Figure 4A) shows an area under the curve (AUC) value of 0.75 with a sensitivity of 70.0% and a specificity of 66.7%.

EXAMPLE 4

Predictive power of biomarker panel

[0067] The predictive power of a panel comprising the putative biomarkers KV110, NAMPT, COPB, SPR2A and HV311 (Figure 5) for early stage heart failure was assessed using MSstats (Clough *et al.*, 2012; Chang *et al.*, 2012), which is based on R (R Development Core Team, 2011). The sensitivity and specificity of the combination of biomarkers in the various cohorts (NYHA Class I, n=20; NYHA Class III/IV, n=19; healthy controls, n=20) are set out in Table 4.

Table 4. Sensitivity and specificity of the combination of biomarkers

	AUC	Sensitivity	Specificity	Positive Predictive Value (PPV)	Negative Predictive Value (NPV)
Class I vs Controls	0.96	95.0 %	90.0 %	94.7 %	90.5 %
Class III/IV vs Controls	0.85	79.0 %	95.0 %	82.6 %	93.8 %
Class III/IV vs Class I	0.65	73.8 %	60.0 %	70.6 %	63.7 %

[0068] The ROC curves in Figure 6 provide a useful summary of the diagnostic potential of the combination of five biomarkers, KV110, NAMPT, COPB, SPR2A and HV311. The closer the area under a ROC curve is to 1, the better the diagnostic potential. The ROC curve for the combination of five biomarkers in NYHA Class I patients compared to the five biomarkers in healthy controls has an AUC of 0.96, a sensitivity of 95.0 % and a specificity of 90.0 % (Figure 6). These results are indicative of high diagnostic capability of the combination of five biomarkers.

[0069] The predictive power of a panel comprising the putative biomarkers KLK1, TCPD, S10A7, DLDH, IGHA2 and CAMP (Figure 7) for early stage heart failure was assessed using MSstats (Clough *et al.*, 2012; Chang *et al.*, 2012), which is based on R (R Development Core Team, 2011). The sensitivity and specificity of the combination of biomarkers in the various cohorts (NYHA Class I, n=20; NYHA Class III/IV, n=19; healthy controls, n=20) are set out in Table 5.

Table 5. Sensitivity and specificity of the combination of biomarkers

	AUC	Sensitivity	Specificity	PPV	NPV
Class I vs Controls					
Class III/IV vs Controls	0.91	84.2%	85.0%	85.0%	84.2%
Class III/IV vs Class I	0.71	68.4%	70%	70.0%	68.5%

[0070] The ROC curves in Figure 8 provide a useful summary of the diagnostic potential of

the combination of six biomarkers, KLK1, TCPD, S10A7, DLDH, IGHA2 and CAMP. The closer the area under a ROC curve is to 1, the better the diagnostic potential. The ROC curve for the combination of six biomarkers in NYHA Class I patients compared to the six biomarkers in healthy controls has an AUC of 0.86, a sensitivity of 80.0 % and a specificity of 70.0 % (Figure 8). These results are indicative of high diagnostic capability of the combination of six biomarkers.

[0071] The predictive power of a panel comprising the putative biomarkers KLK1, S10A7 and CAMP (Figure 9) for individuals with high risk of developing heart failure was assessed using MSstats (Clough *et al.*, 2012; Chang *et al.*, 2012), which is based on R (R Development Core Team, 2011). The sensitivity and specificity of the combination of biomarkers in the various cohorts (heart failure patient, n=100; individuals with high risk of developing heart failure (SCREEN-HF), n=121; healthy controls, n=88) are set out in Table 6.

Table 6. Sensitivity and specificity of the combination of biomarkers

	AUC	**Sensitivity**	**Specificity**	**PPV**	**NPV**
SCREEN-HF vs Controls					
HF patients vs Controls	0.78	73.0%	72.7%	70.3%	75.3%

[0072] Prediction scores between study subjects who developed cardiovascular disease after enrolment in the study, and those who have no cardiovascular disease-related hospital admission are shown in Figure 10.

[0073] Of the 99 participants in the SCREEN-HF cohort, 11 of them were admitted to hospital with cardiovascular diseases as the primary diagnosis. The prediction score generated by the three-marker panel in these 11 individuals ranged from 0.139 to 0.996 with a medium of 0.517 (IQR: 0.256 – 0.920), while in the individuals who did not have cardiovascular disease-related hospital admission, the prediction score ranged from 0.086 to 0.992 with a medium of 0.294 (IQR: 0.172 – 0.679). There is a statistical significant difference between the two groups of SCREEN-HF cohorts (p=0.0382).

[0074] To validate KLK1, TCPD, S10A7, DLDH, IGHA2 and CAMP as members of a diagnostic panel, western blotting analysis was performed on 6 randomly chosen healthy control and 6 randomly chosen heart failure patients. As shown in Figure 11, S10A7 and IGHA2 were

detected in individual saliva samples. S10A7 was detected in 5 of the 6 heart failure patients' samples and only 1 of the 6 healthy control samples. Band intensity of each sample was normalized against the average band intensity of the healthy controls. Similar to the results from SWATH-MS, both S10A7 and IGHA2 demonstrated higher protein abundance in the heart failure patient samples compared to in the healthy control samples. The average band intensity of S10A7 in heart failure patients was 6 times higher than it was in the healthy control samples. IGHA2 has a higher abundance in heart failure patient samples compared to healthy control samples (1.06:1) but no significant different was observed. In contrast to findings in the initial screening, the expression of KLK1 in healthy control and patient samples was similar (1:0.98). CAMP expression was also different, with higher expression in heart failure patients than control (1:1.452). TCPD and DLDH were not detected with western blotting.

[0075] Reference throughout this specification to 'one embodiment' or 'an embodiment' means that a particular feature, structure, or characteristic described in connection with the embodiment is included in at least one embodiment of the present invention. Thus, the appearance of the phrases 'in one embodiment' or 'in an embodiment' in various places throughout this specification are not necessarily all referring to the same embodiment. Furthermore, the particular features, structures, or characteristics may be combined in any suitable manner in one or more combinations.

[0076] In compliance with the statute, the invention has been described in language more or less specific to structural or methodical features. It is to be understood that the invention is not limited to specific features shown or described since the means herein described comprises preferred forms of putting the invention into effect. The invention is, therefore, claimed in any of its forms or modifications within the proper scope of the appended claims (if any) appropriately interpreted by those skilled in the art.

[0077] CITATION LIST

[0078] Australian Institute of Health and Welfare 2011. Cardiovascular disease: Australian facts 2011. Cardiovascular disease series. Cat. no. CVD 53. Canberra: AIHW. (http://www.aihw.gov.au/WorkArea/DownloadAsset.aspx?id=10737418530)

[0079] Bailey UM and Schulz BL, Deglycosylation systematically improves N-glycoprotein identification in liquid chromatography-tandem mass spectrometry proteomics for analysis of cell wall stress responses in Saccharomyces cerevisiae lacking aAlg3p, *J Chromatogr B Analyt Technol Biomed Life Sci*, 2013;923-924:16-21

[0080] Carlson KJ, Lee DCS, Goroll AH, Leahy M and Johnson RA, An analysis of physicians' reasons for prescribing long-term digitalis therapy in outpatients, *J Chron Dis*, 1985;38:733-739

[0081] Castagnola M, Inzitari R, Fanali C, Iavarone F, Vitali A, Desiderio C, Vento G, Tirone C, Romagnoli C, Cabras T, Manconi B, Sanna MT, Boi R, Pisano E, Olianas A, Pellegrini M, Nemolato S, Heizmann CW, Faa G and Messana I, The surprising composition of the salivary proteome of preterm human newborn, *Mol Cell Proteomics*, 2011;10(1):M110.003467

[0082] Chang CY, Picotti P, Hüttenhain R, Heinzelmann-Schwarz V, Jovanovic M, Aebersold R and Vitek O, Protein significance analysis in selected reaction monitoring (SRM) measurements, *Mol Cell Proteomics*, 2012;11(4):M111.014662

[0083] Clough T, Thaminy S, Ragg S, Aebersold R and Vitek O, Statistical protein quantification and significance analysis in label-free LC-MS experiments with complex designs, *BMC Bioinformatics*, 2012;13(Suppl 16):S6

[0084] Foo JYY, Wan Y, Schulz BL, Kostner K, Atherton J, Cooper-White J, Dimeski G and Punyadeera C, Circulating fragments of N-terminal pro-B-type natriuretic peptides in plasma of heart failure patients, *Clin Chem*, 2013;59:1523-1531

[0085] Harlan WR, Oberman A, Grimm R and Rosati RA, Chronic congestive heart failure in coronary artery disease: clinical criteria, *Ann Intern Med*, 1977;86(2):133-138

[0086] Helmerhorst EJ and Oppenheim FG, Saliva: a dynamic proteome, *J Dent Res*, 2007;86:680-693

[0087] Krum H, Jelinek MV, Stewart S, Sindone A and Atherton JJ, 2011 Update to national heart foundation of Australia and cardiac society of Australia and New Zealand guidelines for the prevention, detection and management of chronic heart failure in Australia, 2006, *Med J Aust,* 2011;194(8):405-409

[0088] Loo JA, Yan W, Ramachandran P and Wong DT, Comparative human salivary and plasma proteomes, *J Dent Res*, 2010;89:1016-1023

[0089] McKee PA, Castelli WP, McNamara PM and Kannel WB, The natural history of congestive heart failure: the Framingham study, *N Engl J Med*, 1971;285(26):1441-1446

[0090] Marian AJ and Nambi V, Biomarkers of cardiac disease, *Expert Rev Mol Diagn*, 2004;4:805-20

[0091] Martinet W, Schrijvers DM, De Meyer GRY, Herman AG and Kockx MM, Western array analysis of human atherosclerotic plaques: Downregulation of apoptosis-linked gene 2, *Cardiovasc Res*, 2003;60(2):259-267

[0092] Ovchinnikov DA, Cooper MA, Pandit P, Coman WB, Cooper-White JJ, Keith P, Wolvetang EJ, Slowey PD and Punyadeera C, Tumor-suppressor gene promoter hypermethylation in saliva of head and neck cancer patients, *Transl Oncol*, 2012;5(5):321-326

[0093] Palazzuoli A, Iovine F, Gallotta M and Nuti R, Emerging cardiac markers in coronary disease: Role of brain natriuretic peptide and other biomarkers, *Minerva Cardioangiol*, 2007;55(4):491-496

[0094] Punyadeera C, Dimeski G, Kostner K, Beyerlein P and Cooper-White J, One-step homogeneous C-reactive protein assay for saliva, *J Immunol Methods*, 2011;373:19-25

[0095] R Development Core Team (2011), R: A language and environment for statistical computing, Vienna, Austria: the R Foundation for Statistical Computing

[0096] Shamsham F and Mitchell J, Essentials of the diagnosis of heart failure, *Am Fam Physician,* 2000;61(5):1319-1328

[0097] The Criteria Committee of the New York Heart Association, *Nomenclature and Criteria for Diagnosis of Diseases of the Heart and Great Vessels*, 9th ed., Little, Brown; Boston, 1994, pp. 253-256

CLAIMS

1. A method for detecting early stage heart failure in a subject, the method comprising analysing a biological sample obtained from the subject and determining the concentration of at least one biomarker in the sample and assigning a heart failure classification to the subject if the concentration of the at least one biomarker is either higher or lower than a predefined reference concentration of the at least one biomarker.

2. The method of claim 1, wherein the predefined reference concentration of the at least one biomarker is determined from a biological sample taken from a healthy subject.

3. A method of detecting early stage heart failure in a subject, the method comprising analysing a biological sample obtained from the subject and determining the concentration of at least one biomarker in the sample, determining the concentration of the at least one biomarker in a biological sample obtained from a healthy subject, and assigning a heart failure classification to the subject if the concentration of the at least one biomarker in the sample from the subject is either higher or lower than the concentration of the at least one biomarker in the biological sample obtained from the healthy subject.

4. A method of screening for early stage heart failure in a subject, the method comprising analysing a biological sample obtained from the subject and determining the concentration of at least one biomarker in the sample and assigning a heart failure classification to the subject if the concentration of the at least one biomarker is either higher or lower than a predefined reference concentration of the at least one biomarker.

5. The method of any one of claims 1 to 4, wherein the at least one biomarker is selected from the group of proteins consisting of KLK1, TCPD, S10A7, DLDH, IGHA2, CAMP, KV110, NAMPT, COPB, SPR2A and HV311.

6. The method of claim 5, wherein the at least one biomarker is selected from the group of proteins consisting of KLK1, TCPD, S10A7, DLDH, IGHA2 and CAMP.

7. The method of claim 6, wherein the at least one biomarker is a biomarker panel comprising two, three, four, five or six of the proteins.

8. The method of claim 7, wherein the biomarker panel comprises three of the proteins.

9. The method of claim 8, wherein the biomarker panel comprises KLK1, S10A7, and CAMP.

10. The method of claim 5, wherein the at least one biomarker is selected from the group of proteins consisting of KV110, NAMPT, COPB, SPR2A and HV311.

11. The method of claim 10, wherein the at least one biomarker is a biomarker panel comprising two, three, four or five of the proteins.

12. The method of any one of claims 5 to 11, wherein the biological sample is selected from the group consisting of whole blood, serum, plasma, sputum or saliva.

13. The method of claim 12, wherein the biological sample is saliva.

14. A kit for detecting the presence of at least one biomarker associated with early stage heart failure, the kit comprising a solid support having immobilized thereon at least one molecule that specifically binds to the at least one biomarker.

15. A kit for detecting the presence of at least one biomarker associated with early stage heart failure, wherein the at least one biomarker is selected from the group consisting of KLK1, TCPD, S10A7, DLDH, IGHA2, CAMP, KV110, NAMPT, COPB, SPR2A and HV311, the kit comprising a solid support having immobilized thereon at least one molecule that specifically binds to the at least one biomarker.

16. The kit of claim 14 or claim 15, wherein the at least one molecule that specifically binds to the at least one biomarker is an antibody that specifically binds to the at least one biomarker.

17. The kit of claim 16, wherein the solid support has two, three, four, five or six antibodies immobilized thereon.

18. The kit of claim 17, wherein the solid support has three antibodies immobilized thereon.

19. The kit of claim 18, wherein the antibodies are antibodies to KLK1, S10A7, and CAMP.

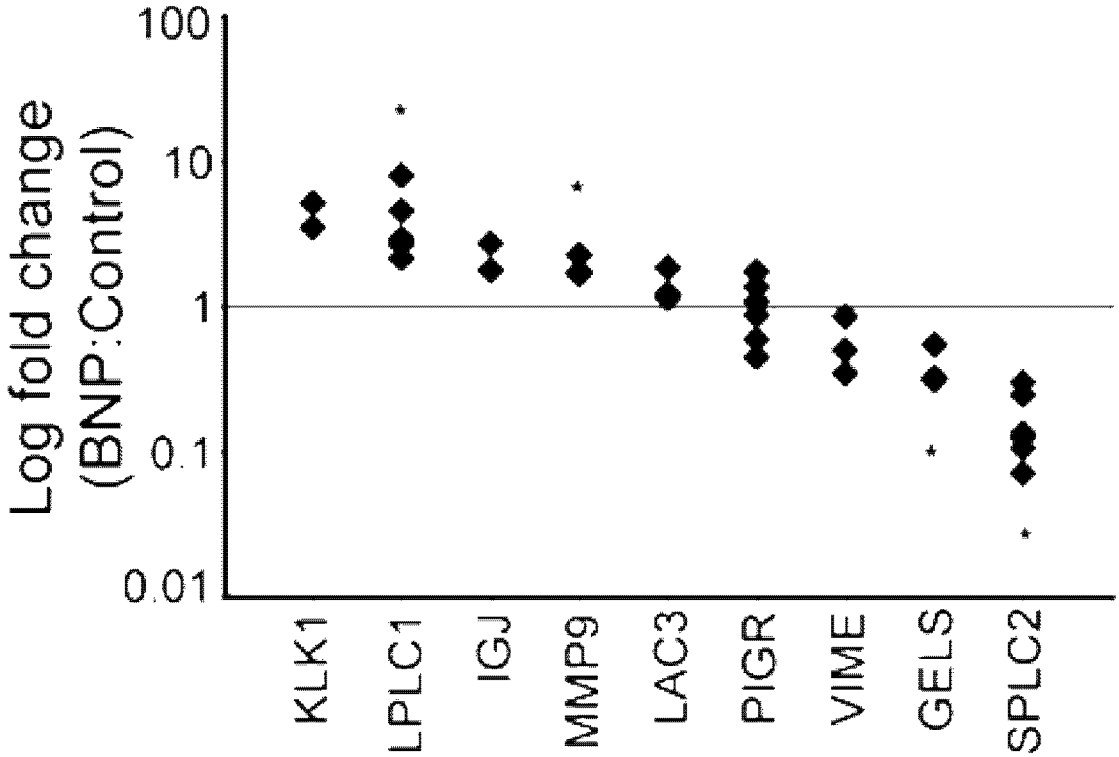

Log fold change (BNP:Control)
100
10
1
0.1
0.01
KLK1
LPLC1
IGJ
MMP9
LAC3
PIGR
VIME
GELS
SPLC2

Figure 1

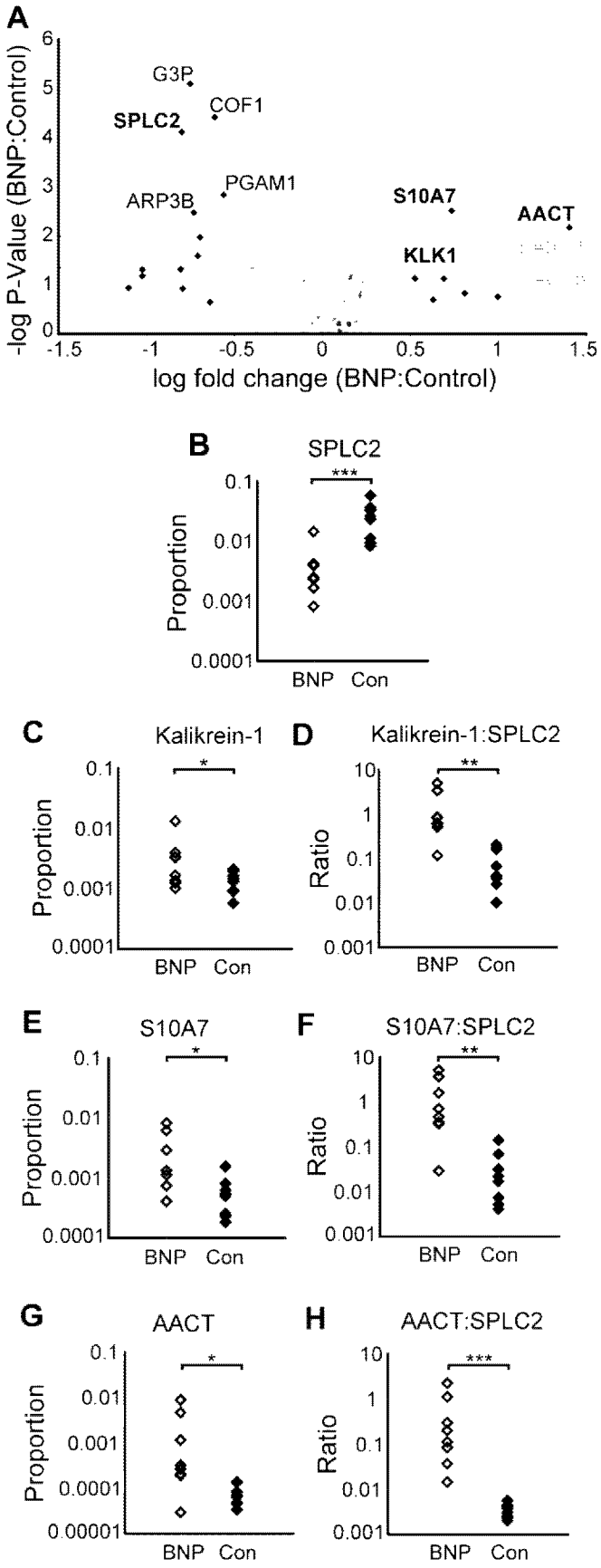

A
-log P-Value (BNP:Control)
log fold change (BNP:Control)
G3P
COF1
SPLC2
PGAM1
ARP3B
S10A7
AACT
KLK1
B
SPLC2
Proportion
BNP Con
C
Kalikrein-1
D
Kalikrein-1:SPLC2
Ratio
E
S10A7
F
S10A7:SPLC2
G
AACT
H
AACT:SPLC2

Figure 2

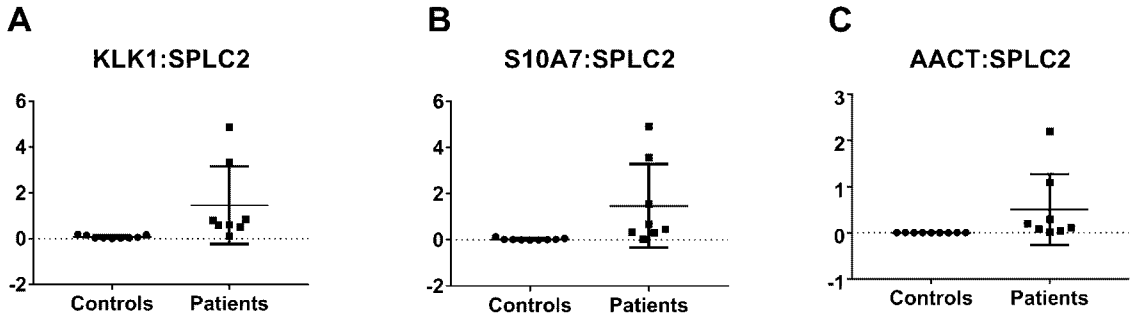


Figure 3

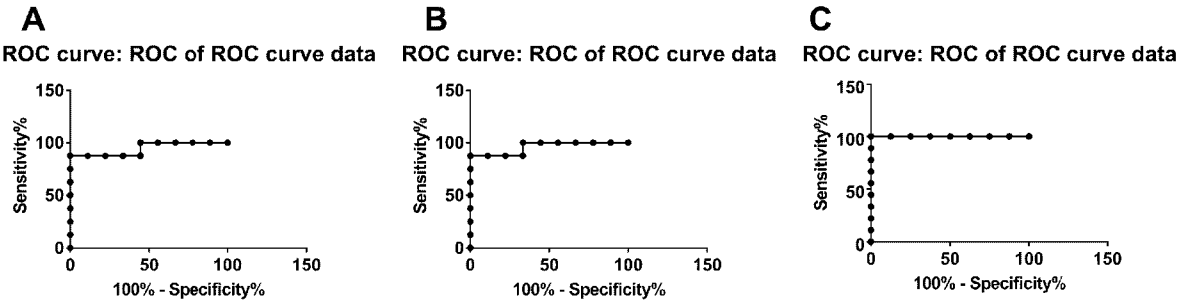


Figure 4

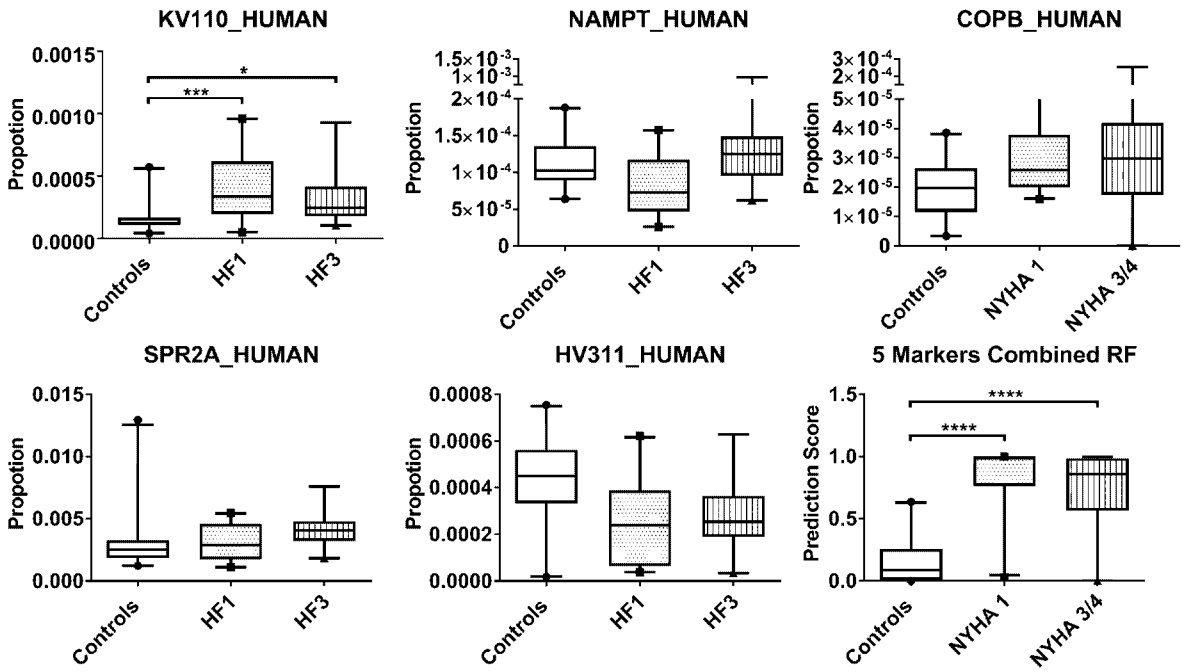


Figure 5

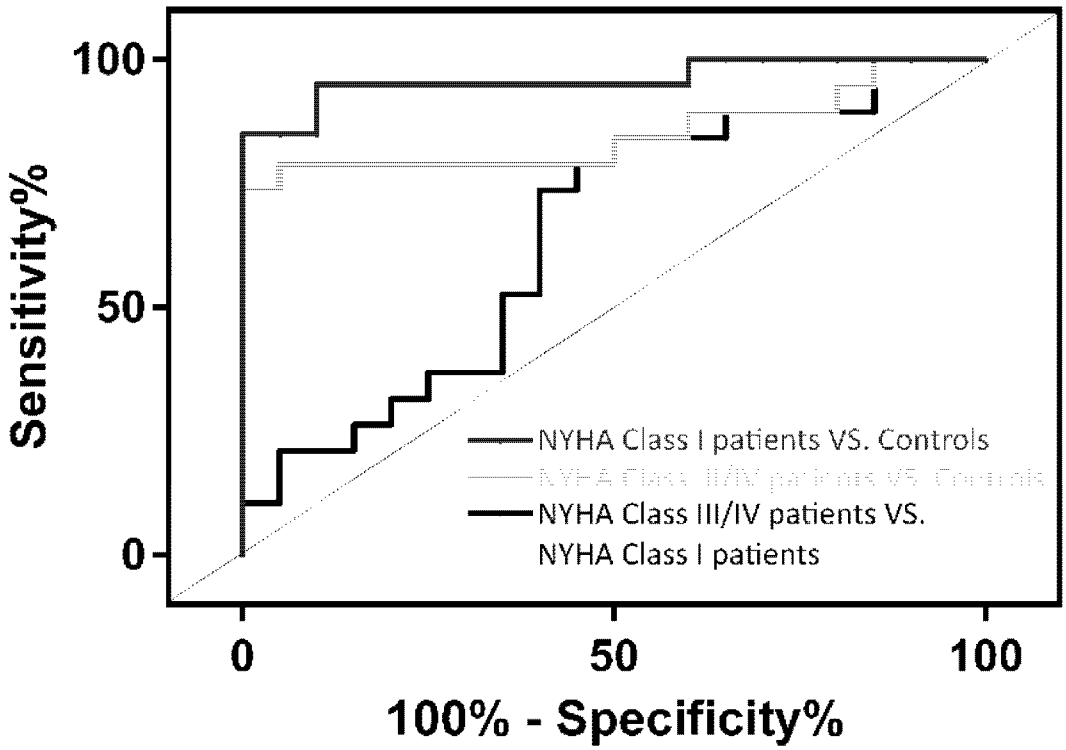


Figure 6

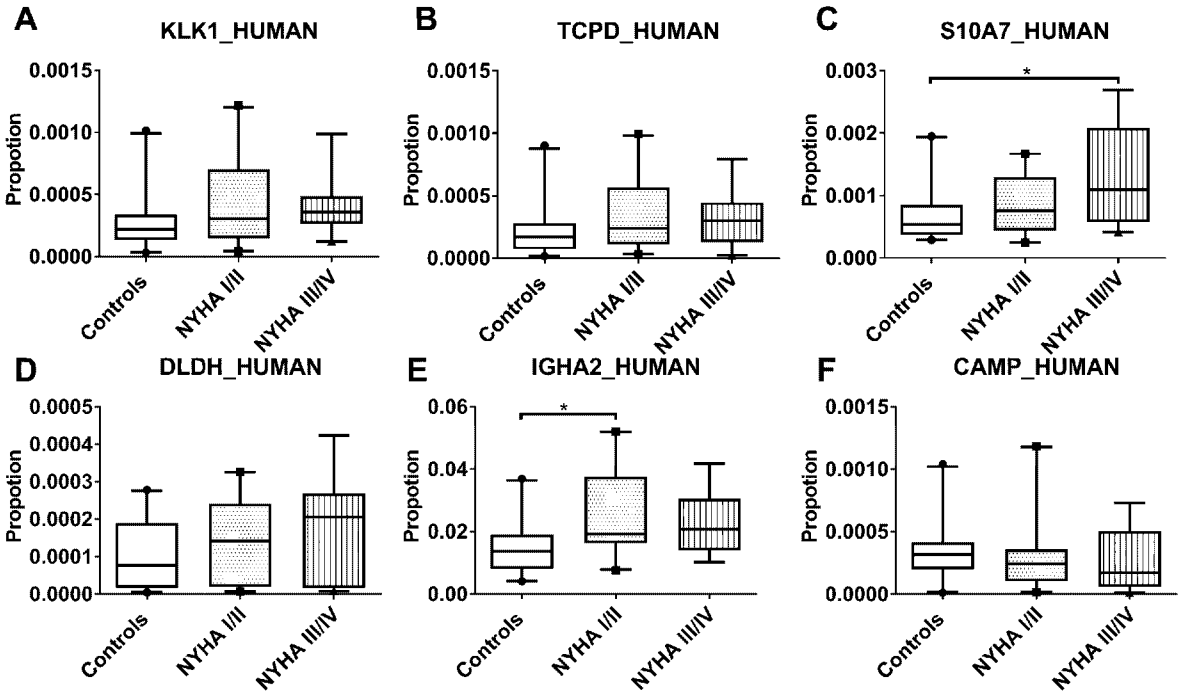


Figure 7

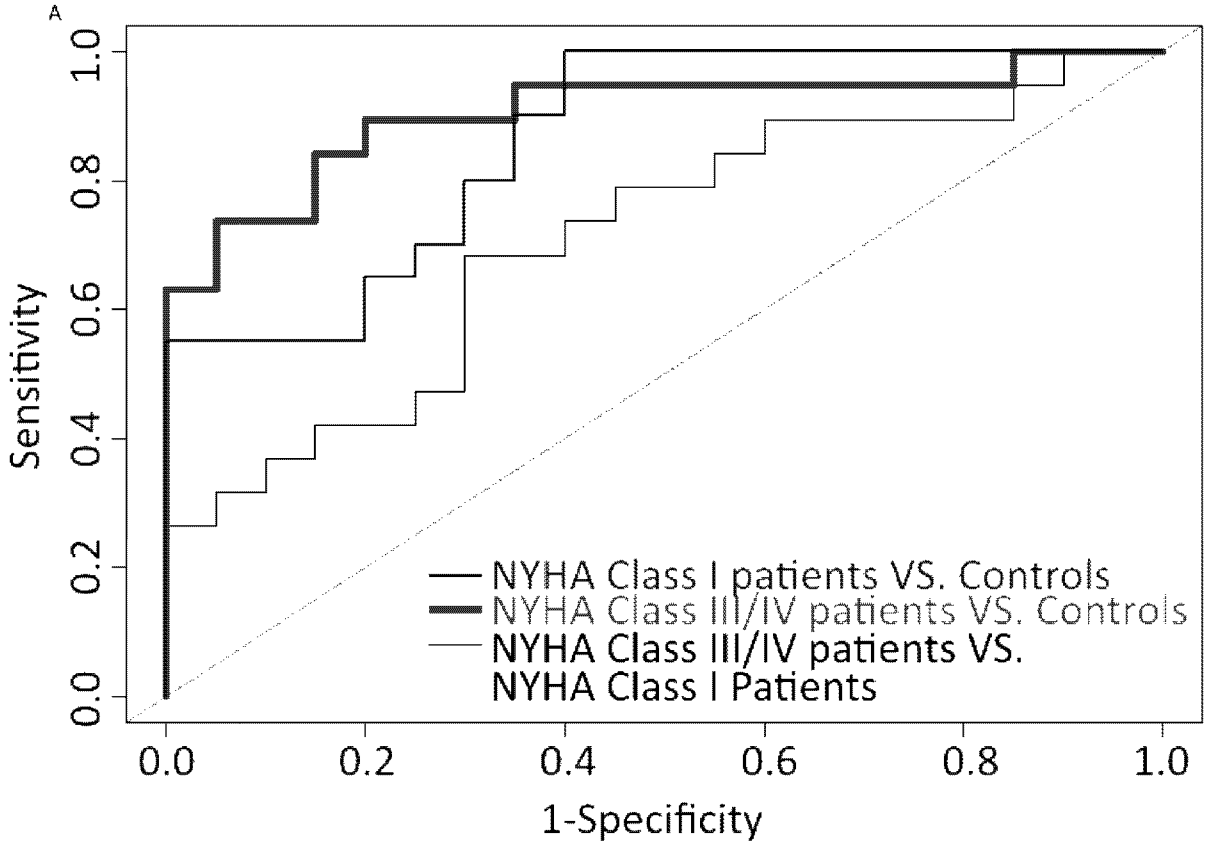

A
Sensitivity
0.0
0.2
0.4
0.6
0.8
1.0
1-Specificity
NYHA Class I patients VS. Controls
NYHA Class III/IV patients VS. Controls
NYHA Class III/IV patients VS.
NYHA Class I Patients

Figure 8

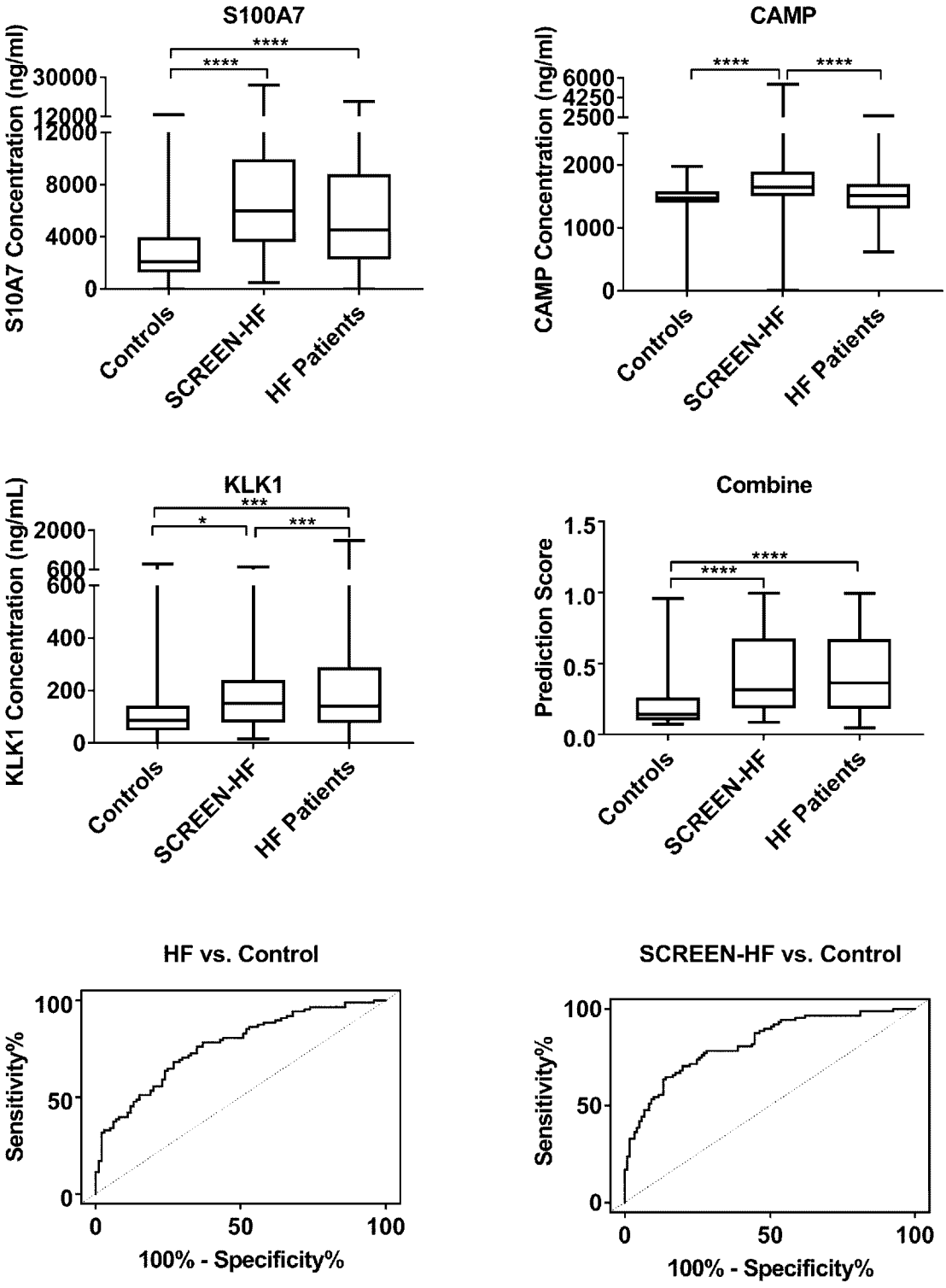

S100A7
S10A7 Concentration (ng/ml)
30000
12000
12000
8000
4000
0
****
****
Controls
SCREEN-HF
HF Patients
CAMP
CAMP Concentration (ng/ml)
6000
4250
2500
2000
1000
0
****
****
Controls
SCREEN-HF
HF Patients
KLK1
KLK1 Concentration (ng/mL)
2000
600
600
400
200
0
***
*
***
Controls
SCREEN-HF
HF Patients
Combine
Prediction Score
1.5
1.0
0.5
0.0
****
****
Controls
SCREEN-HF
HF Patients
HF vs. Control
Sensitivity%
100
50
0
0
50
100
100% - Specificity%
SCREEN-HF vs. Control
Sensitivity%
100
50
0
0
50
100
100% - Specificity%

Figure 9

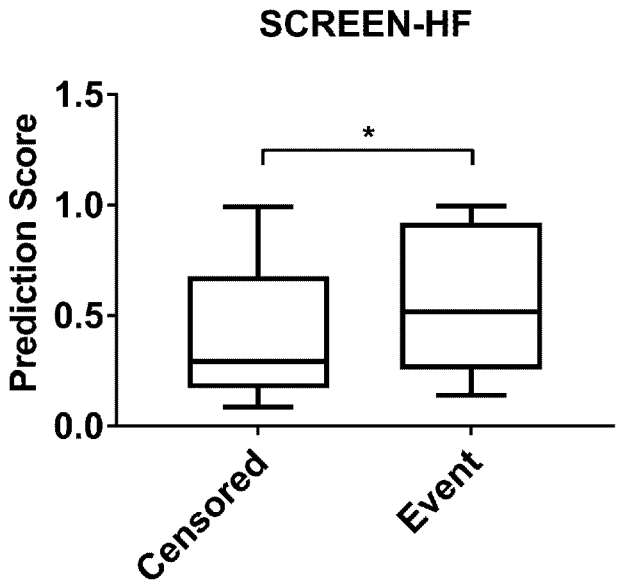
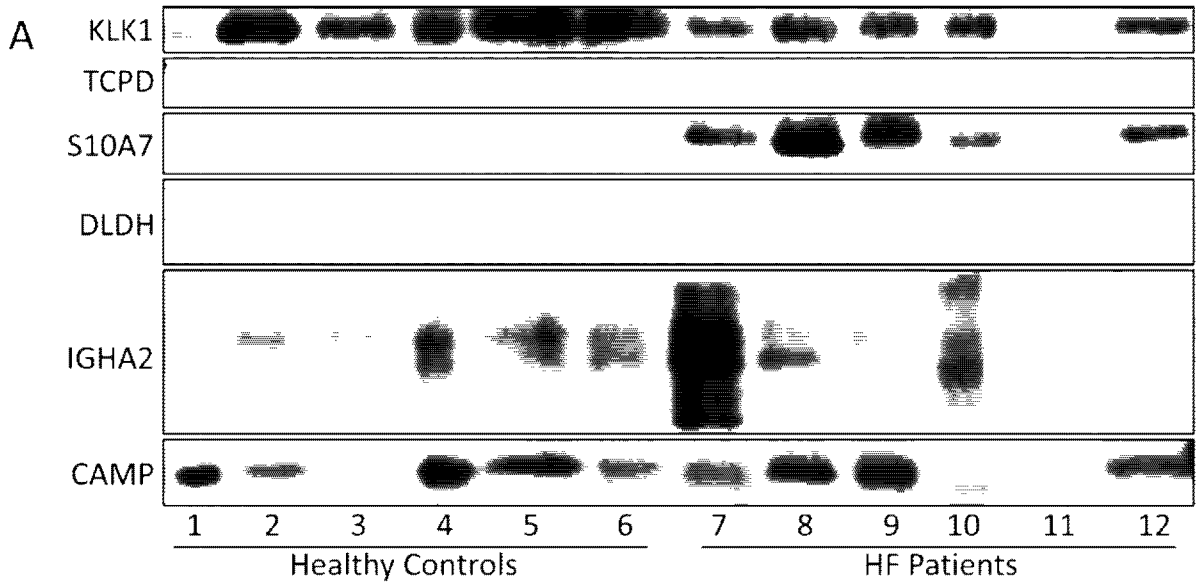


Figure 10


A
KLK1
TCPD
S10A7
DLDH
IGHA2
CAMP
1 2 3 4 5 6 7 8 9 10 11 12
Healthy Controls
HF Patients

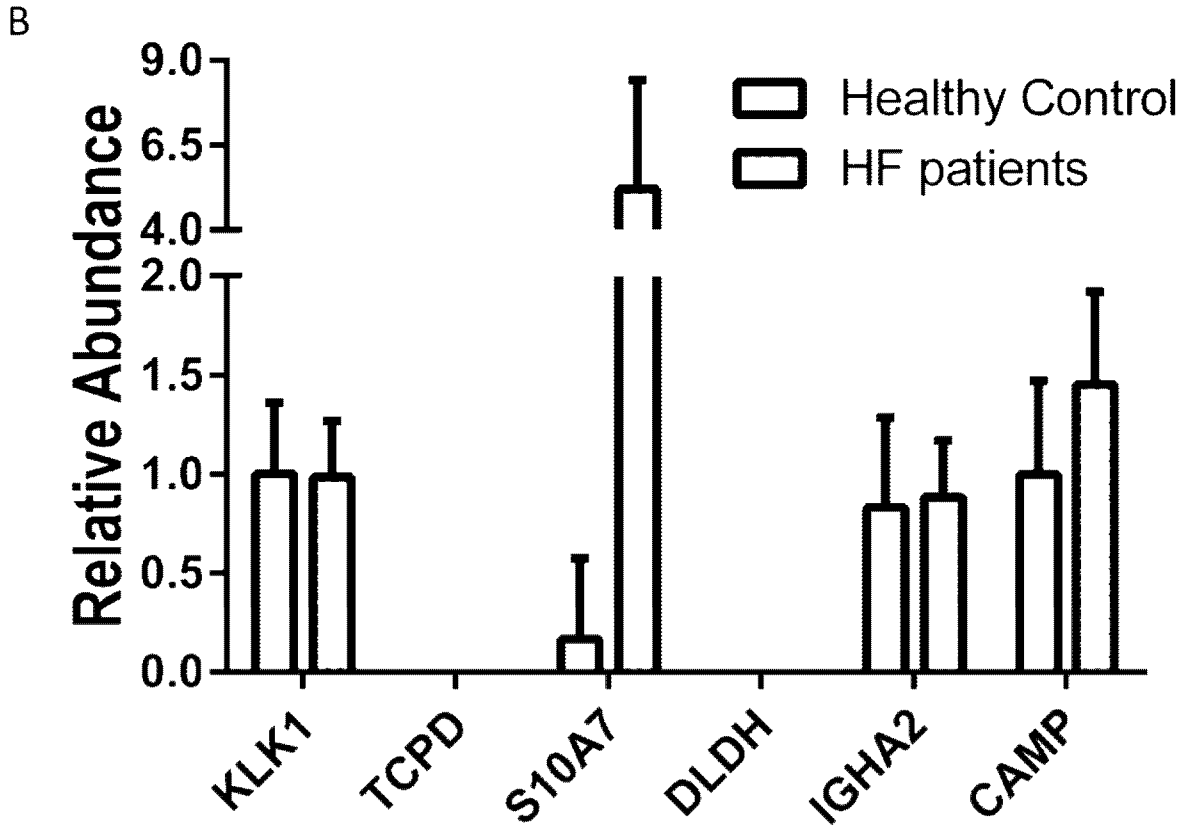

B
Relative Abundance
9.0
6.5
4.0
2.0
1.5
1.0
0.5
0.0
Healthy Control
HF patients
KLK1
TCPD
S10A7
DLDH
IGHA2
CAMP

Figure 11

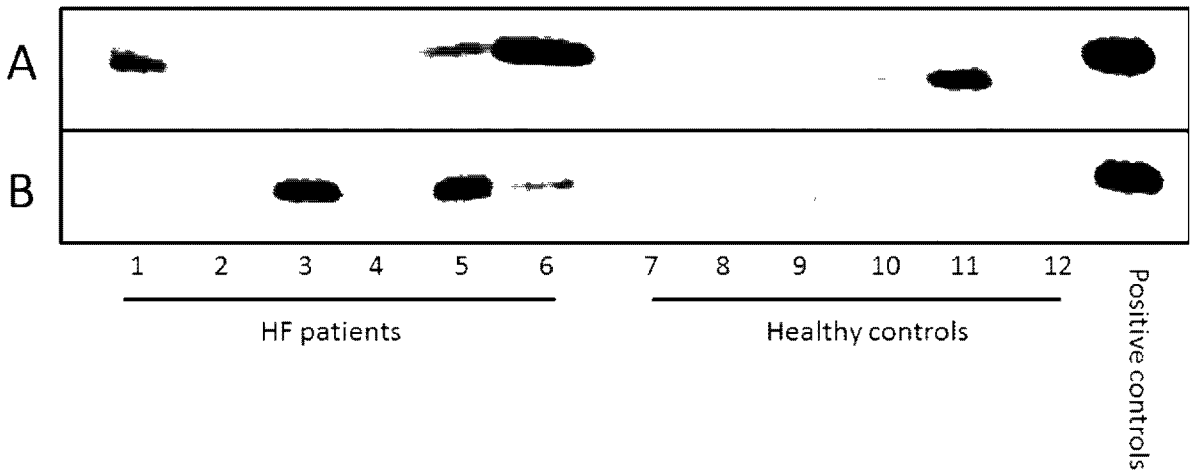

A
B
1
2
3
4
5
6
7
8
9
10
11
12
HF patients
Healthy controls
Positive controls

Figure 12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2018/050827

A. CLASSIFICATION OF SUBJECT MATTER

G01N 33/68 (2006.01) G01N 33/48 (2006.01) G01N 33/50 (2006.01)

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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

PATENW, CAPlus, BIOSIS, MEDLINE, EMBASE - search terms used: heart failure, biomarkers, KLK1, S10A7, cathelicidin antimicrobial peptide, or like terms.

IPC/CPC: G01N33/48, G01N33/50, G01N33/68, G01N2800/32, G01N2800/50

Esp@cenet, Pubmed - Applicant/Inventor search.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 October 2018	02 October 2018

Name and mailing address of the ISA/AU	**Authorised officer**
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustralia.gov.au	Jacky Wong AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. +61262832540

INTERNATIONAL SEARCH REPORT C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		International application No. **PCT/AU2018/050827**
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	IQBAL, N., et al, "Cardiac biomarkers: new tools for heart failure management", Cardiovascular Diagnosis and Therapy, 2012, Vol. 2, No. 2, pp 147-164 Abstract; Figure 1; Table 2; pages 151 and 157	1-8, 10-14, 16-18
X	WO 2016/134365 A1 (THE JOHNS HOPKINS UNIVERSITY) 25 August 2016 Page 3, page 22, page 35; Table 9; claims 8 and 10	14-19
X	WO 2013/090811 A1 (THE JOHNS HOPKINS UNIVERSITY) 20 June 2013 Page 26; Example 3; claims 1, 9, 30, 46	14-19
X	US 2015/0045245 A1 (BIOCARTIS NV) 12 February 2015 Paragraphs [0152], [0153]	14-19
X	WO 2007/148078 A1 (LIPOPEPTIDE AB) 27 December 2007 Page 41	14-19
X	CN 105987998 A (ZONHON BIOPHARMA INST INC, et al.) 05 October 2016 Abstract; claim 1	14-19
X	WO 2011/133770 A2 (BOARD OF REGENTS OF THE UNIVERSITY OF TEXAS SYSTEM) 27 October 2011 Claim 18	14-19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2018/050827

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

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

1. ☐ Claims Nos.:

because they relate to subject matter not required to be searched by this Authority, namely:

the subject matter listed in Rule 39 on which, under Article 17(2)(a)(i), an international search is not required to be carried out, including

2. ☐ Claims Nos.:

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos:

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

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

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

See Supplemental Box for Details

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
Claims 1-19 (Inventions 1-3)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT	International application No. PCT/AU2018/050827

Supplemental Box

Continuation of: **Box III**

This International Application does not comply with the requirements of unity of invention because it does not relate to one invention or to a group of inventions so linked as to form a single general inventive concept.

This Authority has found that there are different inventions based on the following features that separate the claims into distinct groups:

Inventions 1-3: Claims 1-19 (all in part) are directed to methods of detecting early stage heart failure comprising determining the concentration of protein biomarkers selected from KLK1, S10A7 or CAMP in a biological sample from a subject, and kits for said biomarkers; wherein each distinct biomarker represents a separate invention.

Inventions 4-11: Claims 1-8, 10-18 (all in part) are directed to methods of detecting early stage heart failure comprising determining the concentration of a biomarker selected from TCPD, DLDH, IGHA2, KV110, NAMPT, COPB, SPR2A and HV311 in a biological sample from a subject, and kits for detecting said biomarkers; wherein each distinct biomarker represents a separate invention.

PCT Rule 13.2, first sentence, states that unity of invention is only fulfilled when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding special technical features. PCT Rule 13.2, second sentence, defines a special technical feature as a feature which makes a contribution over the prior art.

When there is no special technical feature common to all the claimed inventions there is no unity of invention.

In the above groups of claims, the identified features may have the potential to make a contribution over the prior art but are not common to all the claimed inventions and therefore cannot provide the required technical relationship. The only feature common to all of the claimed inventions and which provides a technical relationship among them is a biomarker for the detection of early stage heart failure. However, this feature does not make a contribution over the prior art because it is disclosed in D1.

D1 discloses various biomarkers for the early diagnosis of heart failure, including B-type natriuretic peptide, N-terminal pro B-type natriuretic peptide, mid-regional pro A-type natriuretic peptide, neutrophil gelatinase-associated lipocalin, high sensitivity troponin and procalcitonin (see Abstract, Figure 1, Table 2, page 157 column 1 second paragraph). The document also discloses the presence of growth differentiation factor-15 in early heart failure (see page 151 second column last paragraph).

Therefore in the light of these documents this common feature cannot be a special technical feature. Therefore there is no special technical feature common to all the claimed inventions and the requirements for unity of invention are consequently not satisfied *a posteriori*.

The applicant's attorney was contacted by telephone and email on 18 September 2018 and confirmed that the applicant did not wish to pay additional fees, and that for the fee already paid the ISA should carry out search and examination on Inventions 1-3. No Form ISA/206 was issued.

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/AU2018/050827**

This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
WO 2016/134365 A1	25 August 2016	WO 2016134365 A1	25 Aug 2016
		EP 3259594 A1	27 Dec 2017
		US 2018217162 A1	02 Aug 2018
WO 2013/090811 A1	20 June 2013	WO 2013090811 A1	20 Jun 2013
		US 2015072360 A1	12 Mar 2015
US 2015/0045245 A1	12 February 2015	US 2015045245 A1	12 Feb 2015
		EP 2788371 A2	15 Oct 2014
		WO 2013083781 A2	13 Jun 2013
WO 2007/148078 A1	27 December 2007	WO 2007148078 A1	27 Dec 2007
		AU 2007262776 A1	27 Dec 2007
		CA 2655022 A1	27 Dec 2007
		EP 2032152 A1	11 Mar 2009
		JP 2009541287 A	26 Nov 2009
		US 2010056431 A1	04 Mar 2010
CN 105987998 A	05 October 2016	CN 105987998 A	05 Oct 2016
		CN 105987998 B	29 Dec 2017
WO 2011/133770 A2	27 October 2011	WO 2011133770 A2	27 Oct 2011
		US 2013116343 A1	09 May 2013

End of Annex

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.