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(54) **Title**: BLOOD GLUTATHIONE AS A BIOMARKER FOR SCREENING ASYMPTOMATIC PATIENTS AT RISK FOR HEART FAILURE

(57) **Abstract**: The present invention relates to a method for screening an asymptomatic patient at risk for heart failure, said method comprising measuring the concentration of glutathione in a blood sample obtained from said patient.

BLOOD GLUTATHIONE AS A BIOMARKER FOR SCREENING ASYMPTOMATIC PATIENTS AT RISK FOR HEART FAILURE

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FIELD OF THE INVENTION:

The present invention relates to a method for screening an asymptomatic patient at risk for heart failure. More particularly, the method of the invention comprises measuring glutathione in a blood sample obtained from said patient.

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BACKGROUND OF THE INVENTION:

Heart failure occurs when the heart is damaged from diseases such as high blood pressure, a heart attack, poor blood supply to the heart, a defective heart
15 valve, atherosclerosis, rheumatic fever, heart muscle disease and so on. The failing heart becomes inefficient, resulting in fluid retention and shortness of breath, fatigue and exercise intolerance. Heart failure is defined by the symptom complex of dyspnea, fatigue and depressed left ventricular systolic function (ejection fraction < 35-40%), and is the ultimate endpoint of all forms of serious heart disease.

20 Despite considerable advances in treatment, heart failure remains associated with high morbidity and mortality [1-3]. Heart failure has many causes and pathophysiological origins. For example, population at risk for developing heart failure include patients with ischaemic heart disease, previous myocardial infarction, atrial fibrillation, hypertension, diabetes, coronary artery disease or obesity, elderly
25 and individuals with a genetic disease. Prevalence and incidence rates of heart failure are growing, and it is presently a leading cause of hospitalization and death in developed countries, but will also likely become a major public health burden for developing countries [4].

Due to the seriousness of heart failure, identifying the individuals at risk of this
30 syndrome is a necessity. Furthermore, screening and early diagnosis of patients at risk for heart failure is extremely desirable since this would make it possible to adapt a faster, easier and less expensive therapeutic monitoring.

For now, routine periodic assessment of LV function in all asymptomatic patients cannot be recommended [4]. Biomarkers for the prognosis of the progression

from an asymptomatic state to symptomatic heart failure are thus needed. A number of biomarkers for heart failure diagnosis and prognosis have been identified [5], which are from diverse biochemical groups and include brain natriuretic peptide (BNP), amino-terminal pro-brain natriuretic peptide (NT-pro BNP), norepinephrine, troponins,
5 heart-type fatty acid binding proteins, myosin light chain- 1, matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinases (TIMPs), C-reactive protein, tumour necrosis factor alpha (TNF-alpha), soluble tumour necrosis factor receptor-1 (TNFR1), soluble IL-2 receptor, and uric acid.

Blood levels of proinflammatory molecules, including sTNF and its receptors
10 sTNFR-1 and -2, are elevated in heart failure patients of NYHA II to IV classes, and are highly predictive of adverse outcomes [29-32]. However, neither TNF nor TNFR-1 or -2 tests do help to screening asymptomatic patients.

B-type natriuretic peptide (BNP) and the amino-terminal fragment of its precursor hormone (NT-pro-BNP) have also received considerable attention as
15 potential screening tests for symptomatic and asymptomatic heart disease. They are secreted by the hemodynamically stressed heart mainly in response to myocardial stretch induced by volume overload. It is of general agreement that these peptides are markers of increased risk of death and hospitalization in symptomatic heart failure patients, and death in acute coronary syndromes [3]. Accordingly, the use of
20 BNP peptides as adjuncts to clinical diagnosis of heart failure is generally admitted, when patient have dyspnea, NT-proBNP being preferred because of its longer half-life [29]. In asymptomatic patients, BNP peptides could be normal or subnormal and the heart failure diagnosis could be mislead. Furthermore, the low specificity limits the clinical utility of BNP peptides as a general screening tool [33,34]. In fact, increased
25 plasma BNP peptides is not unique to heart failure patients, but is also seen in patients with edematous disorders, such as renal failure or ascitic liver cirrhosis, that lead to increased atrial tension or central blood volume.

Therefore the methods based current biomarkers as above described fail to screen the asymptomatic patients at risk for heart failure.

30 Inflammation and oxidative stress are key components in the pathophysiology and progression of heart failure [6-8], and are strongly associated with the disease severity [9-11].

The tripeptide glutathione (L-γ glutamyl-cysteinyl-glycine) does not only play a cardinal role in the maintenance of the cell redox status and defence against

oxidative stress [12], but is also essential in many other cell functions, including cell survival [13]. Recent studies have given evidence that glutathione deficiency determines the adverse effects of tumor necrosis factor-alpha (TNF), exacerbating TNF receptor-1 (TNFR1) apoptotic and negative inotropic effects in isolated cardiomyocytes [14,15], and promoting cardiac remodelling in hypertensive and post-myocardial infarction heart failure rats [16,17]. Furthermore it has been found that the left ventricle (LV) of end-stage heart failure patients is depleted by 54% in glutathione, compared with healthy control LV [17], and depletion in blood glutathione has been reported in advanced heart failure patients with dilated cardiomyopathy [18].

However, no study has yet examined whether blood glutathione level might be related with parameters characterizing heart failure, including the NYHA functional classification (New York Heart Association Functional Classification), LV function and a blood soluble recognized biomarker of heart failure severity (e.g. soluble TNFR1).

SUMMARY OF THE INVENTION:

The invention relates to a method for screening an asymptomatic patient at risk for heart failure said method comprising measuring glutathione in a blood sample obtained from said patient.

The invention also relates to a method for classifying a patient at risk for heart failure, wherein said method comprises the steps of:

(i) measuring the concentration of glutathione in a blood sample obtained from said patient,

(ii) comparing the concentration of glutathione measured in step (i) to a reference value derived from the concentration of glutathione in blood samples from patients who are at particular stages of heart failure or to a control value derived from the concentration of glutathione in blood samples from healthy patients.

The invention relates to a kit for screening an asymptomatic patient at risk for heart failure wherein said kit comprises means for measuring the concentration of glutathione in a blood sample obtained from said patient.

The invention also relates to the use of blood glutathione as a biomarker for screening asymptomatic patients at risk for heart failure.

Finally, the invention relates to N-acetylcysteine for the prevention of heart failure in a patient wherein said patient has been screened or classified according to the methods described above.

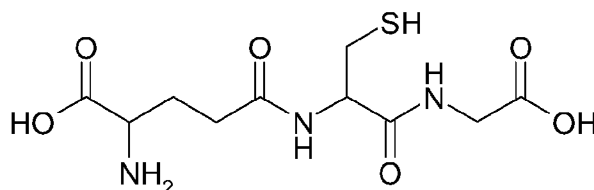
DETAILED DESCRIPTION OF THE INVENTION:

The inventors demonstrate that deficiency in blood glutathione is related with heart failure severity in cardiac patients. Therefore measuring blood glutathione deficiency in a patient may represent a screening test for patients at risk for heart failure. These findings also substantiate the indication of N-acetylcysteine as a complementary therapy for the management of patients at risk for heart failure and displaying glutathione deficiency.

Diagnostic methods and kits:

The present invention relates to a method for screening an asymptomatic patient at risk for heart failure, said method comprising measuring the concentration of glutathione in a blood sample obtained from said patient.

As used herein the term “glutathione” has its general meaning in the art and refers to the total, oxidized and reduced forms of the tripeptide L-γ glutamyl-cysteinyl-glycine which, in its reduced form, has the formula of:



The term "blood sample" as used herein refers to a blood sample (e.g. whole blood sample, serum sample, or plasma sample) obtained for the purpose of in vitro evaluation.

As used herein, the term “patient” denotes a mammal, such as a rodent, a feline, a canine, and a primate. In a preferred embodiment of the invention, a patient according to the invention is a human.

5 According to a particular embodiment, the patient has been affected with a cardiac and/or vascular disease. For example, the patient may be diagnosed with a genetically linked cardiovascular disease, hypertension (high blood pressure), pulmonary hypertension, aortic and mitral valve disease (e.g. stenosis), aortic coarctation, coronary disorders, chronic arrhythmias (e.g. atrial fibrillation), 10 cardiomyopathy of any cause, coronaropathy, valvulopathy or cardiac fibrosis. In another particular embodiment, the patient may be at risk for heart failure because of diabetes, obesity, aging, smoking, dyslipidemia, intoxication or a genetic disease.

The term “asymptomatic patient” refers to a patient who has been classified as a NYHA class I patient. Functional classification of heart failure is generally done by 15 the New York Heart Association Functional Classification (Criteria Committee, New York Heart Association. Diseases of the heart and blood vessels. Nomenclature and criteria for diagnosis, 6th ed. Boston: Little, Brown and co, 1964;114). This classification stages the severity of heart failure into 4 classes (I-IV). The classes (I-IV) are:

20 Class I: no limitation is experienced in any activities; there are no symptoms from ordinary activities.

Class II: slight, mild limitation of activity; the patient is comfortable at rest or with mild exertion.

Class III: marked limitation of any activity; the patient is comfortable only at 25 rest.

Class IV: any physical activity brings on discomfort and symptoms occur at rest.

Once the blood sample from the patient is prepared, the concentration of 30 glutathione may be measured by any known method in the art.

For example, the concentration of glutathione may be measured by using standard enzymatic assay according to the method of [19], as previously used by [16], and as recently updated by [20]. Enzymatic assays utilize glutathione reductase for the quantification of glutathione. For example the sulfhydryl group of glutathione reacts

with DTNB (5,5'-dithio-bis-2-nitrobenzoic acid, Ellman's reagent) and produces a yellow colored 5-thio-2-nitrobenzoic acid (TNB). The disulfide, glutathione that is produced, is then reduced by glutathione reductase to recycle the glutathione and produce more TNB. The rate of TNB production is directly proportional to this recycling reaction which in turn is directly proportional to the concentration of glutathione in the sample. Measurement of the absorbance of TNB at 405 or 412 nm provides therefore an accurate estimation of glutathione in the sample. Many enzymatic assays are commercially available from Alpco Diagnostics, Assay Designs/Stressgen Bioreagents, BioAssay Systems, BioChain, BioVision, Calbiochem, Cayman Chemical, GenScript Corporation, Immundiagnostik, Kamiya Biomedical Company, MBL International, Millipore Corporation, Molecular Probes (Invitrogen), Neogen Corporation, Novagen, Oxis International, Inc., Promega, Sigma-Aldrich, and Thermo Scientific Pierce Protein Research Products.

Other methods include standard electrophoretic and immunodiagnostic techniques, including immunoassays such as competition, direct reaction, or sandwich type assays. Such assays include, but are not limited to, Western blots; agglutination tests; enzyme-labeled and mediated immunoassays, such as ELISAs; biotin/avidin type assays; radioimmunoassays; immunoelectrophoresis; immunoprecipitation, high performance liquid chromatography (HPLC), size exclusion chromatography, solid-phase affinity, etc.

In a particular embodiment, the methods of the invention comprise contacting the blood sample with a binding partner capable of selectively interacting with glutathione in said blood sample.

The binding partner may be generally an antibody that may be polyclonal or monoclonal, preferably monoclonal. Polyclonal antibodies directed against glutathione can be raised according to known methods by administering the appropriate antigen or epitope to a host animal selected, e.g., from pigs, cows, horses, rabbits, goats, sheep, and mice, among others. Various adjuvants known in the art can be used to enhance antibody production.

Monoclonal antibodies against glutathione can be prepared and isolated using any technique that provides for the production of antibody molecules by continuous cell lines in culture. Techniques for production and isolation include but are not limited to the hybridoma technique originally described by [21]; the human B-cell hybridoma technique [22]; and the EBV-hybridoma technique [23]. Alternatively,

techniques described for the production of single chain antibodies (see e.g. U.S. Pat. No. 4,946,778) can be adapted to produce anti-glutathione, single chain antibodies. Antibodies useful in practicing the present invention also include anti-glutathione fragments including but not limited to F(ab')2 fragments, which can be generated by
5 pepsin digestion of an intact antibody molecule, and Fab fragments, which can be generated by reducing the disulfide bridges of the F(ab')2 fragments. Alternatively, Fab and/or scFv expression libraries can be constructed to allow rapid identification of fragments having the desired specificity to glutathione. For example, phage display of antibodies may be used. In such a method, single-chain Fv (scFv) or Fab
10 fragments are expressed on the surface of a suitable bacteriophage, e. g., M13. Briefly, spleen cells of a suitable host, e. g., mouse, that has been immunized with a protein are removed. The coding regions of the VL and VH chains are obtained from those cells that are producing the desired antibody against the protein. These coding regions are then fused to a terminus of a phage sequence. Once the phage is
15 inserted into a suitable carrier, e. g., bacteria, the phage displays the antibody fragment. Phage display of antibodies may also be provided by combinatorial methods known to those skilled in the art. Antibody fragments displayed by a phage may then be used as part of an immunoassay.

Antibodies against glutathione may be commercially available from Abcam,
20 AbD Serotec, Abgent, Abnova Corporation, ABR-Affinity BioReagents, Acris Antibodies GmbH, Advanced Targeting Systems, Assay Designs/Stressgen Bioreagents, Atlas Antibodies, Aviva Systems Biology, BioGenex, Biosensis, Calbiochem, Cayman Chemical, Epitomics, Inc., Everest Biotech, GeneTex, GenScript Corporation, GenWay Biotech, Inc., HyTest Ltd., IMGENEX, Lab Vision,
25 Lifespan Biosciences, MBL International, Millipore Corporation, Novus Biologicals, ProSci, Inc, Proteintech, Group, Inc., QED Bioscience Inc., R&D Systems, Raybiotech, Inc., Rockland Immunochemicals, Inc., Santa Cruz Biotechnology, Inc.,ScyTek Laboratories, and Tocris Bioscience,.

In another embodiment, the binding partner may be an aptamer. Aptamers are
30 a class of molecule that represents an alternative to antibodies in term of molecular recognition. Aptamers are oligonucleotide or oligopeptide sequences with the capacity to recognize virtually any class of target molecules with high affinity and specificity. Such ligands may be isolated through Systematic Evolution of Ligands by EXponential enrichment (SELEX) of a random sequence library, as described in [24].

The random sequence library is obtainable by combinatorial chemical synthesis of DNA. In this library, each member is a linear oligomer, eventually chemically modified, of a unique sequence. Possible modifications, uses and advantages of this class of molecules have been reviewed in [25]. Peptide aptamers consist of
5 conformationally constrained antibody variable regions displayed by a platform protein, such as E. coli Thioredoxin A, that are selected from combinatorial libraries by two hybrid methods [26].

The binding partners of the invention such as antibodies or aptamers, may be labelled with a detectable molecule or substance, such as a fluorescent molecule, a
10 radioactive molecule or any others labels known in the art. Labels are known in the art that generally provide (either directly or indirectly) a signal.

As used herein, the term "labeled", with regard to the antibody, is intended to encompass direct labeling of the antibody or aptamer by coupling (i.e., physically linking) a detectable substance, such as a radioactive agent or a fluorophore (e.g.
15 fluorescein isothiocyanate (FITC) or phycoerythrin (PE) or Indocyanine (Cy5)) to the antibody or aptamer, as well as indirect labeling of the probe or antibody by reactivity with a detectable substance. An antibody or aptamer of the invention may be labeled with a radioactive molecule by any method known in the art. For example radioactive molecules include but are not limited radioactive atom for scintigraphic studies such
20 as I123, I124, In111, Re186, Re188.

The aforementioned assays generally involve the bounding of the binding partner (ie. Antibody or aptamer) in a solid support. Solid supports which can be used in the practice of the invention include substrates such as nitrocellulose (e. g., in membrane or microtiter well form); polyvinylchloride (e. g., sheets or microtiter wells);
25 polystyrene latex (e.g., beads or microtiter plates); polyvinylidine fluoride; diazotized paper; nylon membranes; activated beads, magnetically responsive beads, and the like.

More particularly, an ELISA method can be used, wherein the wells of a microtiter plate are coated with a set of antibodies against glutathione. A blood
30 sample containing or suspected of containing glutathione is then added to the coated wells. After a period of incubation sufficient to allow the formation of antibody-antigen complexes, the plate(s) can be washed to remove unbound moieties and a detectably labeled secondary binding molecule added. The secondary binding molecule is allowed to react with any captured sample marker protein, the plate

washed and the presence of the secondary binding molecule detected using methods well known in the art.

Glutathione can be determined by Nuclear Magnetic Resonance Spectroscopy.

5 Measuring the concentration of glutathione may also include separation of the proteins: centrifugation based on the protein's molecular weight; electrophoresis based on mass and charge; HPLC based on hydrophobicity; size exclusion chromatography based on size; and solid-phase affinity based on the protein's affinity for the particular solid-phase that is use. Once separated, glutathione may be 10 identified based on the known "separation profile" e. g., retention time, for that protein and measured using standard techniques. Alternatively, the separated proteins may be detected and measured by, for example, a mass spectrometer.

Another further object of the invention relates to a method for screening an 15 asymptomatic patient at risk for heart failure, said method comprising the steps of:

(i) measuring the concentration of glutathione in a blood sample obtained from said patient,
(ii) comparing the concentration of glutathione measured in step (i) to a control value derived from the concentration of glutathione in blood 20 samples from healthy patients

wherein a decreased concentration of glutathione in the blood sample obtained from said patient as compared to said control value indicates that the patient is at risk for heart failure.

25 For example, the concentration of glutathione in the blood sample of a patient can be deemed to be decreased when it is less than 2mM, preferably less than 1.9mM, even more preferably less than 1.8mM, 1.7mM, 1.6mM or 1.5mM.

Another further object of the invention relates to a method for classifying a 30 patient at risk for heart failure, wherein said method comprises measuring the concentration of glutathione in a blood sample obtained from said patient.

In a particular embodiment, said method further comprises the steps of:

(i) measuring the concentration of glutathione in a blood sample obtained from said patient,

(ii) comparing the concentration of glutathione measured in step (i) to a reference value derived from the concentration of glutathione in blood samples from patients who are at particular stages of heart failure and/ or to a control value derived from the concentration of glutathione in blood samples from healthy patients.

In a further embodiment of the invention, methods of the invention comprise measuring the concentration of at least one further biomarker.

The term "biomarker", as used herein, refers generally to a molecule, the expression of which in a blood sample from a patient can be detected by standard methods in the art (as well as those disclosed herein), and is predictive or denotes a condition of the subject from which it was obtained.

For example, the other biomarker may be selected from the group heart failure biomarkers consisting of brain natriuretic peptide (BNP), amino-terminal pro-brain natriuretic peptide (NT-pro BNP), norepinephrine, troponin, heart-type fatty acid binding protein, myosin light chain-1, matrix metalloproteinase, tissue inhibitor of matrix metalloproteinase, C-reactive protein (CRP), TNFalpha, soluble tumor necrosis factor receptor 1 (sTNFR1), soluble TNFR2 receptor, soluble IL-2 receptor, CD40-CD154, CCAM-I, P-selectin, tissue factor and von Willebrand factor, urocortin , myeloperoxidase, and uric acid.

In a preferred embodiment, the further biomarker of heart failure is NT-pro BNP or sTNFR1.

Yet another object of the invention relates to a kit for performing a method of the invention, said kit comprising means for measuring the concentration of glutathione in a blood sample obtained from a patient. The kit may include means for the performance of the enzymatic methods as described above such as glutathione reductase and DTNB. The kit may alternatively include an antibody, or a set of antibodies as above described. In a particular embodiment, the antibody or set of antibodies are labelled as above described. The kit may also contain other suitably packaged reagents and materials needed for the particular detection protocol, including solid-phase matrices, if applicable, and standards. The kit may also contain one or more means for the detection of a further biomarker. Typically the kit may also contain means for the detection of one ore more heart failure biomarker selected

from the group consisting of brain natriuretic peptide (BNP), amino-terminal pro-brain natriuretic peptide (NT-pro BNP), norepinephrine, troponin, heart-type fatty acid binding protein, myosin light chain-1, matrix metalloproteinase, tissue inhibitor of matrix metalloproteinase, C-reactive protein (CRP), TNFalpha, soluble tumor
5 necrosis factor receptor 1 (sTNFR1), soluble T2 receptor, soluble IL-2 receptor, CD40-CD154, CCAM-I, P-selectin, tissue factor and von Willebrand factor, urocortin, myeloperoxidase, and uric acid.

In a preferred embodiment, kit of the invention comprises means for measuring the concentration of glutathione and means for measuring the
10 concentration of NT-pro BNP and/or sTNFR1.

A further object of the invention relates to the use of blood glutathione as a biomarker for screening an asymptomatic patient at risk for heart failure.

15 ***Therapeutic methods:***

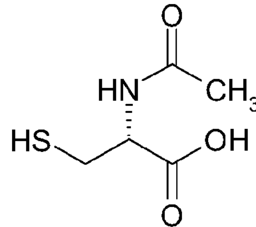
The method of the invention may be thus useful for screening or classifying patients at risk for heart failure and then may be used to choose the accurate treatment. For example, patients with a low level of glutathione may receive a more
20 intensive treatment and attention compared to patient with higher level. Such method may thus help the physician to make a choice on a prophylactic treatment, which can accordingly consist in administering accurate drugs to the patients. Costs of the treatments may therefore be adapted to the severity and morbidity of the patients.

As used herein, the term “prevention” refers to preventing the disease or
25 condition from occurring in a subject which has not yet been diagnosed as having it.

Accordingly a further object of the invention relates to a method for preventing heart failure in a patient comprising a step of screening said patient at risk for heart failure according to any method of the invention and a step of administering said patient with the accurate therapeutic drugs and regimen.

30 For example, drugs that may be useful for the prevention of heart failure may be selected from the group consisting of ACE inhibitors, beta-blockers and aldosterone antagonists.

In a preferred embodiment, the drug is selected as having the capability to restore the glutathione level in the heart. For example, said drug may be the glutathione precursor N-acetylcysteine (NAC) which has the formula of:



5 Accordingly, a further object of the invention relates to N-acetylcysteine (NAC) for the prevention of heart failure in a patient wherein said patient has been screened or classified according to one of the methods described above.

In a particular embodiment, the invention relates to a method for preventing heart failure in a patient, said method comprising the steps of:

10 i) screening or classifying said patient at risk for heart failure according to any method of the invention

ii) administering said patient with an effective amount of NAC.

By a "effective amount of NAC" is meant a sufficient amount of NAC to prevent heart failure at a reasonable benefit/risk ratio applicable to any medical treatment. It 15 will be understood, however, that the total daily usage of NAC will be decided by the attending physician within the scope of sound medical judgment.

The specific therapeutically effective dose level for any particular patient will thus depend upon the severity of the disorder. Other factors may will also impact the therapeutically dose level such as the specific composition employed, the age, body 20 weight, general health, sex and diet of the patient; the time of administration, route of administration, and rate of excretion of NAC; the duration of the treatment; drugs used in combination or coincidental with NAC; and like factors well known in the medical arts. For example, it is well within the skill of the art to start doses of the compound at levels lower than those required to achieve the desired therapeutic 25 effect and to gradually increase the dosage until the desired effect is achieved.

Finally, methods of the invention may be applied for monitoring the therapeutic outcome of a patient who has been screened or classified at risk for heart failure.

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

FIGURES:

Figure 1: Relation between LVEF or blood sTNFR1 level and functional NYHA class in patients undergoing cardiac surgery (n=76) and healthy controls (n=15). The cohort displayed a standard relation between LVEF (A) or sTNFR1 (B) and functional NYHA class. sTNFR1 indicated the cleaved extracellular domain of TNFR1. LVEF: left ventricular ejection fraction. Linear trends P<0.0001. *P<0.05 vs healthy controls (C). †P<0.05 vs NYHA class I; ‡P<0.05 vs NYHA class II; §P<0.05 vs NYHA class III.

Figure 2: Relation between atrial tissue glutathione and functional NYHA class in patients undergoing cardiac surgery. Atrial tissue glutathione content was significantly decreased in symptomatic NYHA class IV patients compared with asymptomatic NYHA class I patients. Linear trend P<0.03. *P<0.05 vs NYHA class I.

Figure 3: Atrial tissue glutathione in the subgroups of patients with coronary artery diseases (CAD) or aortic stenosis (AS), according to preserved LVEF (>45%) or LV dysfunction (≤45%). (A) Deficiency in atrial glutathione was related with LV dysfunction in CAD patients. In contrast, atrial glutathione was low in AS patients, independently of the LVEF value. *P<0.05 vs LVEF>45%. (B) In CAD patients, atrial glutathione correlated significantly with the LVEF value (r=0.45, P=0.006).

Figure 4: Blood glutathione in patients undergoing cardiac surgery and in the CAD and AS subgroups of patients. (A) In patients undergoing cardiac surgery, blood glutathione decreased as a function of NYHA class (linear trend P<0.0001). (B) Compared with healthy controls, blood glutathione in the CAD and AS subgroups of patients was depleted, independently of the LVEF value. *P<0.05 vs healthy controls (C); †P<0.05 vs NYHA class I.

Figure 5: Correlation between blood glutathione and blood sTNFR1 in patients undergoing cardiac surgery. The decrease in blood glutathione was exponentially related with the elevation in blood sTNFR1 (r= 0.88).

5 **EXAMPLE:**

Material & Methods:

Patients: The study included 76 patients undergoing cardiac surgery
10 (coronary artery bypass grafting (CABG), aortic valve replacement, orthotopic heart transplantation and ventricular assist device implantation) from 2004 to 2007. Patients with sepsis, endocarditis and renal failure were excluded. Fifteen healthy volunteers with normal left ventricular ejection fraction were recruited by the Centre d'Investigation Clinique of the Hôpital Henri Mondor. Clinical data and transthoracic
15 echocardiographies (Vivid 7, GE, Norway), using American society of echocardiography recommendations [27], were obtained for all patients. Blood samples were obtained from the 15 volunteers. Venous blood samples and right atrial appendages were obtained from patients undergoing cardiac surgery for coronary artery bypass graft or aortic valve replacement with cardiopulmonary bypass. Blood
20 samples only were obtained from patients undergoing left ventricular assist device implantation. Right atrial and venous blood samples were immediately frozen in liquid nitrogen, and stored at -80°C until use. Paroxystic post-surgery atrial fibrillation was recorded.

All patients had given written informed consent before surgical procedures
25 were performed. All studies are conformed to the Declaration of Helsinki and institutional ethical regulations.

Assays for glutathione and sTNFR1: Atrial tissue samples were cut into 20 µm sections. Homogenates were prepared from 5 frozen sections of each sample by
30 homogenization at 4°C, in 200 µl of 50 mM Hepes, pH 7.4, containing protease inhibitors (1mM PMSF, 2 µg/ ml leupeptin, 2 µg/ ml aprotinin), using a tissuelyzer (Qiagen). Glutathione was assayed in atrial homogenates or whole blood, according to a modification of Tietze's method [19] as previously described [16] and as recently

updated [20]. sTNFR1 was quantified in whole blood with ELISA kits (Quantikine, R&D Systems).

Statistical analysis: Results are given as means ± sem. Data were analyzed by Mann-Whitney test or Kruskal-Wallis test and Dunn post test, as appropriate (Prism, GraphPad Software Inc). Differences were considered statistically significant at $P < 0.05$.

Results:

Clinical and biological characteristics of the patients: The mean age of healthy controls was 52±4 years and their mean Left Ventricular Ejection Fraction (LVEF) was 62±1%. The clinical and biological characteristics of the 76 patients undergoing surgery for coronary artery disease (CAD; n=43), aortic valve stenosis (AS; n=25), or dilated cardiomyopathy (n=8, transplantation or mechanical assist device implantation) are reported in Table 1.

The cohort consisted of 22%, 31%, 29% and 18% patients divided into functional NYHA class I, II, III and IV, respectively. Symptomatic patients in NYHA class III and IV displayed a significant decrease in LVEF compared with the control group, whereas asymptomatic patients of NYHA class I had a preserved LV function (Figure 1A). Patients with CAD and patients with AS constituted the two principal groups of our cohort. Compared with CAD patients, AS patients displayed significant hypertrophy of septal (ST) and posterior (PWT) walls and high LVEF, illustrating an aortic stenosis-induced cardiomyopathic remodelling supporting compensation of LV ejection fraction (LVEF) (Table 1).

Mean blood level of sTNFR1 (a recognized marker of heart failure severity) increased by 3- to 5-fold in symptomatic patients of NYHA class II to IV as compared with healthy controls (Figure 1B). Of note, the mean blood sTNFR1 level in our asymptomatic, NYHA class I patients, was not statistically different from control values.

Table 1. Clinical and biological characteristics of patients undergoing cardiac surgery

	All patients (n=76)	Coronary Artery Diseases (CAD) (n=43)	Aortic Stenosis (AS) (n=25)	P Value CAD *vs* AS
Demographic				
Male/ female (n)	60/ 16	37/ 6	15/ 10	0.01
Age, yrs	66 ±1	62 ± 2	75 ± 2	<0.0001
NYHA, mean	2.3 ± 0.1	2.2 ± 0.2	2.6 ± 0.2	NS
I, (%)	22	31	5	
II, (%)	31	31	42	
III, (%)	29	26	37	
IV, (%)	18	12	16	
Clinical characteristics				
Hypertension, (%)	56	64	36	NS
Hypercholesterolemia, (%)	56	81	47	0.03
Diabetes mellitus, (%)	35	45	17	NS
Chronic Arrythmia, (%)	12	5	24	0.01
Echocardiographic data				
LVEF (%)	45.3 ± 2.1	45 ± 2	53 ± 4	0.06
LVEDD (mm)	54.3 ± 1.3	55.4 ± 1.5	50.1 ± 1.8	0.04
iLVEDD ($mm.cm^{-2)}$	30 ± 1	30 ± 1	29 ± 1	NS
ST (mm)	11.6 ± 0.4	10.3 ± 0.4	13.3 ± 0.7	0.0001
PWT (mm)	10.8 ± 0.4	9.8 ± 0.4	12.1 ± 0.6	0.002
LA diameter (mm)	41 ± 2	41 ± 2	42 ± 3	NS
Systolic PAP (mm Hg)	44 ± 2	42 ± 4	43 ± 3	NS
Medications (%)				
Beta-blockers	51	71	22	0.0005
ACE inhibitors	35	50	11	NS
AT-II type 1 receptor antagonists	21	20	22	NS
Diuretics	48	43	57	NS
Aldosterone antagonists	16	20	9	NS
Statin	60	77	35	0.001
Surgery characteristics				
No urgent surgery, n(%)	83	81	100	0.02
Post Operative AF (%)	30	21	53	0.0006
Biochemical data				
CRP (mg/ l)	11 ± 2	11.4 ± 2.6	10.9 ± 6.0	NS
Haemoglobin (g/ dl)	13.1 ± 0.2	13.3 ± 0.3	12.8 ± 0.5	NS
Total bilirubin (mg/ dl)	17 ± 2	17.6 ± 2.6	17.4 ± 2.6	NS
Creatinine (µmol/ l)	106 ± 5	109 ± 8	100 ± 6	NS
Blood glutathione (mM)	1.4±0.1	1.46 ± 0.07	1.23±0.06	0.02
Tissue glutathione (nmol/ mg)	2.0±0.2	2.03 ± 0.23	1.91±0.27	NS
Blood sTNF R1 (ng/ ml)	0.9±0.5	0.79 ± 0.07	0.97 ±0.07	NS

LVEF: left ventricular ejection fraction; LVEDD: left ventricular end diastolic diameter; iLVEDD: indexed LVEDD; ST: end-diastolic septal wall thickness; PWT: end-diastolic posterior wall thickness; LA diameter: left atrial diameter; PAP: pulmonary artery pressure. CRP: C-reactive protein. Post-operative AF: post-operative atrial fibrillation. Post-operative AF excluded patients who were in chronic atrial fibrillation, and patient having heart transplant or left ventricular assist device. In CAD and AS, blood glutathione was not correlated with the age (P = 0.46).

Right atrial Glutathione in patients with heart disease: The mean content of glutathione in right atrial tissue was markedly decreased by 58% in patients of NYHA class IV compared with asymptomatic patients of NYHA class I (1.0 ± 0.2 vs 2.4 ± 0.2 nmol glutathione/ mg tissue, respectively; P = 0.002; Figure 2).

5 Next, we considered separately the two principal groups of CAD and AS patients, distributed into 2 subgroups on the basis of their LV function. The subgroup of CAD patients with LVEF dysfunction (≤45%) displayed 40% larger deficiency in atrial glutathione content than the subgroup with preserved LVEF (>45%) (Figure 3A). In fact, in CAD patients, atrial glutathione was positively correlated with the
10 LVEF (r = 0.45, P = 0.0064; Figure 3B). In contrast, in AS patients, atrial glutathione content was low independently of the LV function, similar to that of CAD patients with LVEF dysfunction (2.0 ± 0.3 *vs* 1.7 ± 0.2 glutathione nmol/ mg tissue) (Figure 3A).

Venous blood glutathione deficiency in patients with cardiac disease:
15 Next, we examined whether deficiency in systemic glutathione affected patients with cardiac diseases. Considering the all cohort of patients, there was no significant correlation between the right atrial and venous blood glutathione concentrations (r = 0.11, P = 0.43). Nevertheless, as compared with healthy controls, mean venous blood level of glutathione was significantly decreased in patients with cardiac
20 diseases (from 20% in NYHA class I to 40% in NYHA class II to IV). And mean venous blood levels of symptomatic patients of NYHA class II to IV were significantly lower than that of asymptomatic patients of NYHA class I (p<0.05, Figure 4A).Venous blood glutathione levels in CAD and AS patients were significantly lower than that of healthy controls, independently of the LVEF value (Figure 4B).
25 Finally, the decrease in blood glutathione level was exponentially correlated with the increase in blood sTNFR1 level (r = 0.88, Figure 5).

Conclusion:

30 The first important finding of this study is that a drop in blood glutathione discriminates asymptomatic NYHA I class patients from healthy controls. This observation brings to light the potential of blood glutathione test for screening asymptomatic individuals at risk for heart failure. As previously stated [28], an improved identification of asymptomatic individuals, through screening and diagnosis, may lead

to the early initiation of appropriate pharmacological therapy. Appropriate therapy, in turn, can improve outcomes and decrease progression to heart failure, morbidity and mortality.

Optimized screening of asymptomatic patients will rely on the combination of independent biomarkers ahead of clinical examination. For example, blood glutathione test can be combined with NT-proBNP. The interest of such a combination relies on the independent information provided by each marker, the one related with vascular injury, and the other one related with oxidative stress and inflammation. The predictive value of the combination of two such markers is much greater than that of each biomarker taken separately.

These data further document the potentiality of oral administration of the glutathione precursor, N-acetylcysteine (NAC), as a complement treatment to current medical therapies for cardiac diseases. Noteworthy, glutathione deficiency will not only weaken cell defence against oxidative stress, but also many other cellular functions including cell survival [36]. Interestingly, a recent study Ozaydin et al. reported that NAC, administered by intravenous infusion for 1h before and 48h after cardiac surgery, decreased the incidence of postoperative atrial fibrillation (AF) [37]. The inventors have discovered that NAC administration to an asymptomatic patient at risk for heart failure can be optimized by performing the method for screening and/or classifying said asymptomatic patient prior to treatment.

In conclusion, our findings bring to light the systemic deficiency in glutathione in asymptomatic patients with structural heart disease. Accordingly, blood glutathione provides a new biomarker for screening asymptomatic patients at risk for heart failure.

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Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

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CLAIMS:

1. A method for screening an asymptomatic patient at risk for heart failure, said method comprising measuring the concentration of glutathione in a blood sample obtained from said patient.

2. The method according claim 1, wherein said method further comprises measuring the concentration of at least one biomarker selected from the group consisting of brain natriuretic peptide (BNP), amino-terminal pro-brain natriuretic peptide (NT-pro BNP), norepinephrine, troponin, heart-type fatty acid binding protein, myosin light chain-1, matrix metalloproteinase, tissue inhibitor of matrix metalloproteinase, C-reactive protein (CRP), TNFalpha, soluble tumor necrosis factor receptor 1 (sTNFR1), soluble TNFR2 receptor, soluble IL-2 receptor, CD40-CD154, CCAM-I, P-selectin, tissue factor and von Willebrand factor, urocortin or uric acid.

3. The method according to claim 2, wherein said method further comprises measuring the concentration of NT-pro BNP and/or sTNFR1.

4. A method for classifying a patient at risk for heart failure, wherein said method comprises the steps of:

   (i) measuring the concentration of glutathione in a blood sample obtained from said patient,

   (ii) comparing the concentration of glutathione measured in step (i) to a reference value derived from the concentration of glutathione in blood samples from patients who are at particular stages of heart failure and/ or to a control value derived from the concentration of glutathione in blood samples from healthy patients .

5. A kit for performing a method according to any one of claims 1 to 4 wherein said kit comprises means for measuring the concentration of glutathione in a blood sample obtained from the patient.

6. The kit according to claim 5 wherein said kit further comprises means for measuring the concentration of least one biomarkers selected from the group consisting of brain natriuretic peptide (BNP), amino-terminal pro-brain natriuretic peptide (NT-pro BNP), norepinephrine, troponin, heart-type fatty
5 acid binding protein, myosin light chain-1, matrix metalloproteinase, tissue inhibitor of matrix metalloproteinase, C-reactive protein (CRP), TNFalpha, soluble tumor necrosis factor receptor 1 (sTNFR1), soluble T2 receptor, soluble IL-2 receptor, CD40-CD154, CCAM-I, P-selectin, tissue factor and von Willebrand factor or urocortin or uric acid.

10 7. The kit according to claim 6, wherein said kit comprises means for measuring the concentration of NT-pro BNP and/or sTNFR1.

8. Use of blood glutathione as a biomarker for screening asymptomatic patients at risk for heart failure.

9. N-acetylcysteine for the prevention of heart failure in a patient wherein said
15 patient has been screened or classified according to the method of any one of claims 1 to 4.

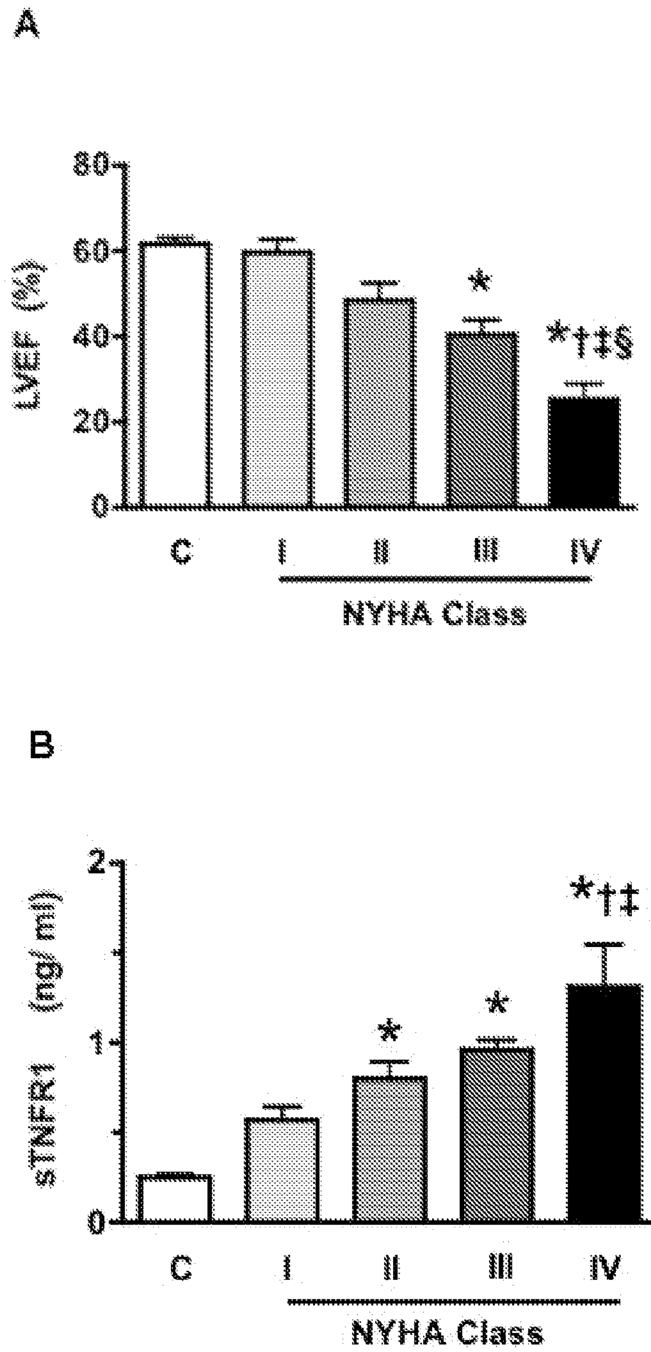

Figure 1

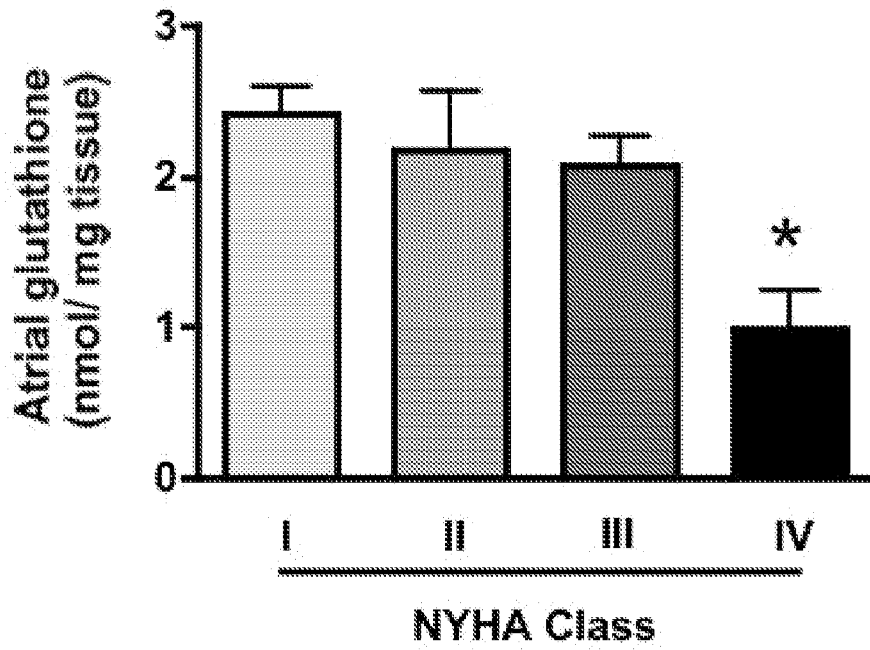

Figure 2

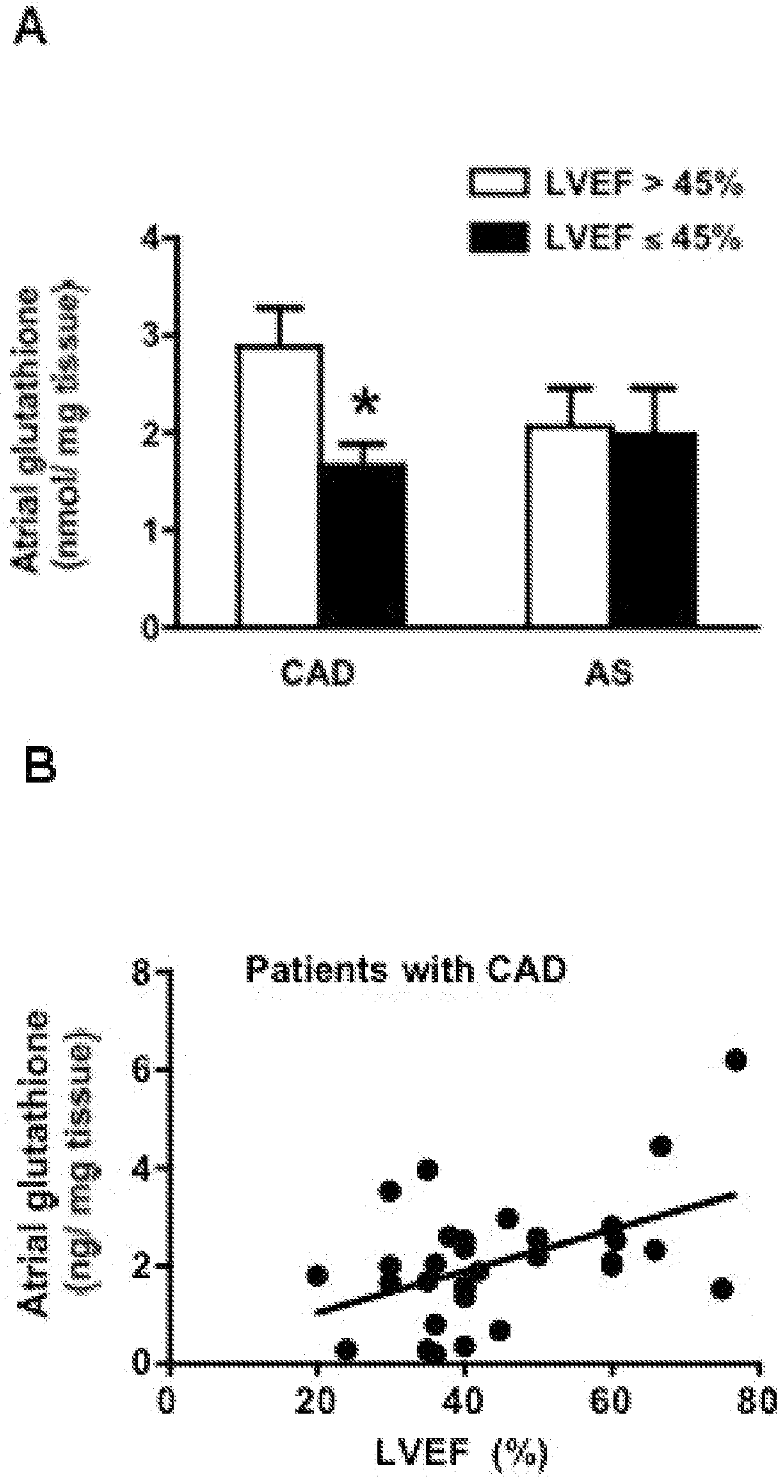

Figure 3

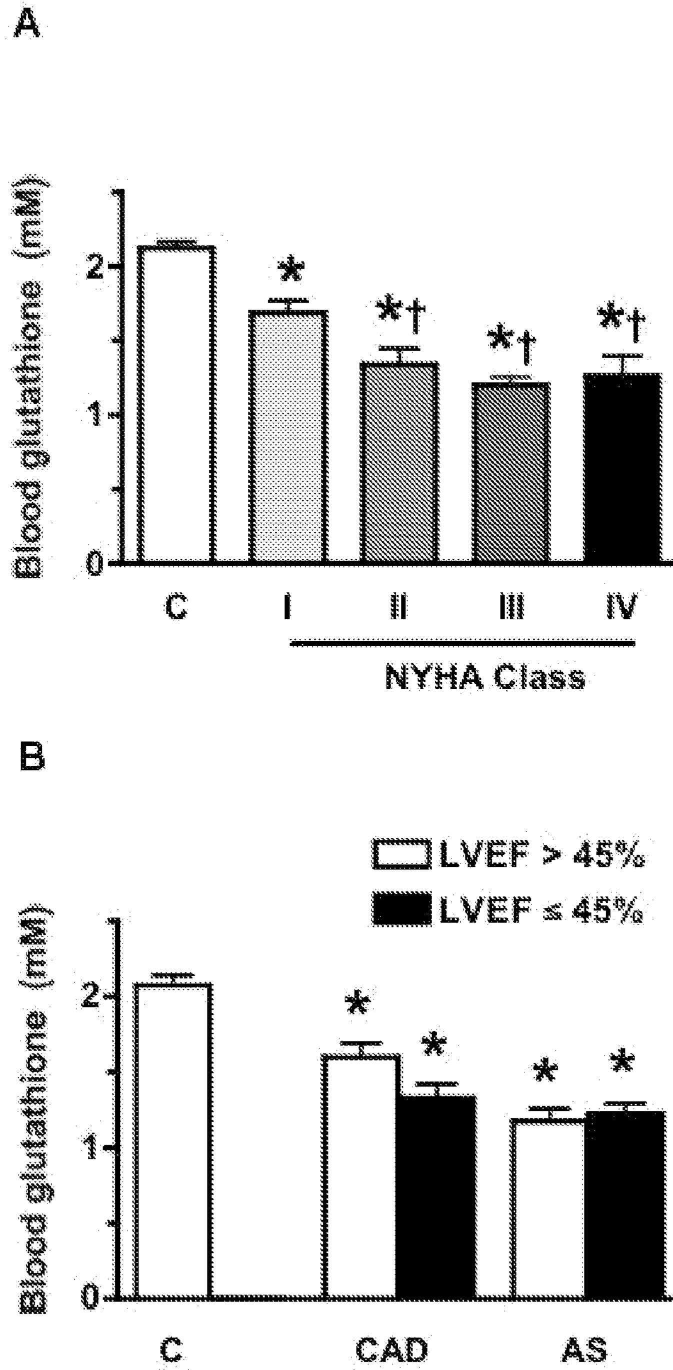

Figure 4

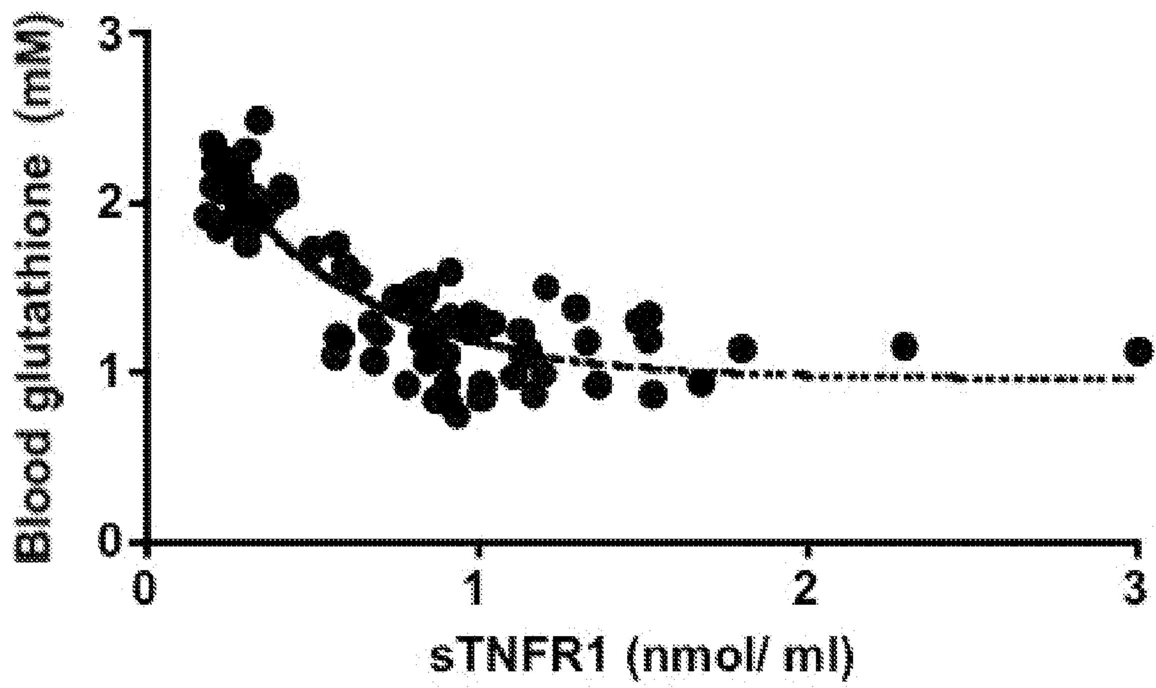

Figure 5

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2009/059242

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/68 G01N33/53

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)
G01N

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 practical, search terms used)

EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2005/046675 A2 (HOLTZMAN JORDAN [US]) 26 May 2005 (2005-05-26) page 1, lines 31-34; page 2, lines 1-5; page 2, lines 26-34; page 3, lines 1-6; page 22, claim 1	9

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[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 document 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
21 September 2009	27/10/2009

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Motrescu-Hateley, E

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ADAMY ET AL: "Neutral sphingomyelinase inhibition participates to the benefits of N-acetylcysteine treatment in post-myocardial infarction failing heart rats" JOURNAL OF MOLECULAR AND CELLULAR CARDIOLOGY, ACADEMIC PRESS, GB, vol. 43, no. 3, 27 August 2007 (2007-08-27), pages 344-353, XP022214894 ISSN: 0022-2828 cited in the application	9
Y	the whole document	1-4,6-8
Y	US 2006/105419 A1 (BLANKENBERG STEFAN [DE] ET AL) 18 May 2006 (2006-05-18) paragraphs 0002, 0004, 0005, 0010, 0011, 0021-0024, 0030, 0031, 0040-0053, 0057, 0070-0075, 0077; page 17, claims 1-6, 13-22, 26, 27.	1-4,6-8
Y	NIELSEN OLAV W ET AL: "Retrospective analysis of the cost-effectiveness of using plasma brain natriuretic peptide in screening for left ventricular systolic dysfunction in the general population." JOURNAL OF THE AMERICAN COLLEGE OF CARDIOLOGY 1 JAN 2003, vol. 41, no. 1, 1 January 2003 (2003-01-01), pages 113-120, XP002503275 ISSN: 0735-1097 the whole document	2,6
Y	AL-MALLAH MOUAZ ET AL: "POSITIVE TROPONIN IN DIABETIC KETOACIDOSIS WITHOUT ACUTE CORONARY SYNDROME PREDICTS POOR OUTCOME" CIRCULATION, AMERICAN HEART ASSOCIATION, DALLAS, TX, vol. 110, no. 17, SUPPL.S, 1 October 2004 (2004-10-01), page 413(ABSTRACT1948), XP008069195 ISSN: 0009-7322 the whole document	2,6
Y	SHARMA RAKESH ET AL: "Elevated circulating levels of inflammatory cytokines and bacterial endotoxin in adults with congenital heart disease." THE AMERICAN JOURNAL OF CARDIOLOGY 15 JUL 2003, vol. 92, no. 2, 15 July 2003 (2003-07-15), pages 188-193, XP002503276 ISSN: 0002-9149 the whole document	2-3,6-7

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	RAUCHHAUS M ET AL: "Plasma cytokine parameters and mortality in patients with chronic heart failure." CIRCULATION 19 DEC 2000, vol. 102, no. 25, 19 December 2000 (2000-12-19), pages 3060-3067, XP002503277 ISSN: 1524-4539 the whole document	2-3,6-7
X	BIOVISION, INC: "ApoGSH tm Glutathione Fluorometric/Colormetric Assays" PRODUCT INFO, [Online] 4 March 2007 (2007-03-04), XP002546616 Retrieved from the Internet: URL:http://web.archive.org/web/20070304054209/http://www.biovision.com/updated/gluthathione.html> [retrieved on 2009-09-21]	5
Y	the whole document	6-7
X	US 2005/202521 A1 (CRUM ALBERT [US]) 15 September 2005 (2005-09-15)	5
Y	paragraphs 0001, 0013-0019,0313-0314, 0210-0211; claim 1.	1-4,6-8

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2009/059242

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2005046675 A2	26-05-2005	NONE	
US 2006105419 A1	18-05-2006	NONE	
US 2005202521 A1	15-09-2005	AR 048031 A1	22-03-2006
		US 2008213905 A1	04-09-2008
		WO 2005086827 A2	22-09-2005

patsnap

专利名称(译)	血液谷胱甘肽作为一种生物标志物，用于筛查有心力衰竭风险的无症状患者		
公开(公告)号	EP2310860A1	公开(公告)日	2011-04-20
申请号	EP2009802484	申请日	2009-07-17
[标]申请(专利权)人(译)	法国国家健康医学研究院		
申请(专利权)人(译)	INSERM - INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE		
当前申请(专利权)人(译)	INSERM - INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE		
[标]发明人	DAMY THIBAUD LE CORVOISIER PHILIPPE PAVOINE CATHERINE PECKER FRANCOISE CARAMELLE PHILIPPE		
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摘要(译)

本发明涉及筛选有心力衰竭风险的无症状患者的方法，所述方法包括测量从所述患者获得的血液样品中谷胱甘肽的浓度。