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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
27 December 2013 (27.12.2013)

WIPO | PCT

(10) International Publication Number
WO 2013/190041 A1

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

(21) International Application Number:
PCT/EP2013/062885

(22) International Filing Date:
20 June 2013 (20.06.2013)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
12305722.6 22 June 2012 (22.06.2012) EP

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(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, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, 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, 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, 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 2013/190041 A1

(54) Title: METHODS AND KITS FOR PREDICTING THE SURVIVAL TIME OF POST ACUTE MYOCARDIAL INFARCTION PATIENTS

(57) Abstract: The present invention relates to a method for predicting the survival time of a post acute myocardial infarction patient comprising i) determining the level of MMP-8 in a blood sample obtained from the patient, ii) comparing the level determined at step i) with a predetermined reference value and iii) providing a bad prognosis when the level determined at step i) is higher than the predetermined reference level and a good prognosis when the level determined at step i) is lower than the predetermine reference value.

METHODS AND KITS FOR PREDICTING THE SURVIVAL TIME OF POST ACUTE MYOCARDIAL INFARCTION PATIENTS

FIELD OF THE INVENTION:

The present invention relates to methods and kits for predicting the survival time of a post acute myocardial infarction patient.

BACKGROUND OF THE INVENTION:

Left ventricular (LV) remodelling after myocardial infarction (MI) is characterized by progressive LV dilatation[1], and is associated with an increased risk of heart failure and cardiovascular death[2]. Recent studies have shown that LV remodelling remains relatively frequent after MI, despite a high rate of acute reperfusion and widespread prescription of secondary prevention medications[3,4]. Although several variables - such as MI size - have been identified as risk factors[5,6], LV remodelling is a process that remains difficult to predict in clinical practice. The concept that biological markers may accurately predict clinical outcome is an attractive one. There is growing interest in the research of biological markers that could be used for patients risk assessement in many medical fields. We previously performed a systematic review of the published evidence about the association of circulating biomarkers with LV remodelling after MI: at least a quarter of associations concerned biomarkers of extracellular matrix (ECM) turnover: matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinases (TIMPs) or collagen peptides[7]. Recently published guidelines for the development of biomarkers have emphasized the importance of the link between underlying pathophysiology and the predictive capabilities of the biomarkers chosen. Accordingly, biomarkers of ECM homeostasis were selected for this study because past experimental studies have shown that changes within the ECM are an integral part of LV remodelling after MI[8]. In a variety of animal models of MI, increased MMP expression occurs during the development of heart failure[9], MMP inhibition attenuates LV remodelling[10,11] and TIMP deficiency accelerates adverse LV remodelling by promoting ECM degradation[12].

However, previous clinical studies investigating the relationships between circulating levels of MMPs/TIMPs and LV remodelling had discordant results. It must be noticed that most studies were of relatively small size. Moreover, timing of LV remodelling evaluation varied greatly among studies. This point is important because LV remodelling is a time-dependent process that has been demonstrated to continue for > 6 to 12 months after MI.

Studies with a shorter follow-up therefore focus only on early remodelling and not on the entire process. Similarly, because expression of biomarkers after acute MI is also time dependent, studies with serial blood samplings are useful to determine the timing of the strongest association between biomarkers and LV remodelling. Previous studies with large cohorts only focused on baseline sampling or investigated limited number of MMPs/TIMPs. We therefore designed the present study to provide an in depth investigation of the relations of circulating MMPs and all TIMPs measured 4 times during one-year follow-up with one-year LV remodelling. MMPs/TIMPs levels were determined using multiplex technology in samples from the REVE-2 study – a study prospectively designed to analyze relationships between circulating biomarkers and LV remodelling in 246 patients after a first anterior MI and in which repeat echocardiographic examinations as well as serial blood sampling were performed during the first year post-MI.

SUMMARY OF THE INVENTION:

The present invention relates to a method for predicting the survival time of a post acute myocardial infarction patient comprising i) determining the level of MMP-8 in a blood sample obtained from the patient, ii) comparing the level determined at step i) with a predetermined reference value and iii) providing a bad prognosis when the level determined at step i) is higher than the predetermined reference level and a good prognosis when the level determined at step i) is lower than the predetermine reference value.

DETAILED DESCRIPTION OF THE INVENTION:

Changes within the extracellular matrix, implicating matrix metalloproteinases (MMP) and tissue inhibitors of metalloproteinases (TIMPs), are an integral part of left ventricular (LV) remodelling after myocardial infarction (MI). The inventors investigated the temporal profile of circulating MMPs and TIMPs and their relationship with LV volumes at 1 year and outcome in post-MI patients. This prospective multicenter study included 246 patients with a first anterior Q-wave MI. Serial echocardiographic studies were performed at hospital discharge and 1 year after MI and analysed at a core laboratory. The clinical endpoint was a composite of cardiovascular death or hospitalization for heart failure episode during 3 years follow-up. Serum samples for determination of MMP-1, -2, -3, -8, -9, -13, TIMP-1,-2, -3, -4 levels were obtained at hospital discharge, 1-month, 3-months, and 1-year and analyzed using multiplex technology. Baseline MMP-8 and MMP-9 were positively associated with LV end-diastolic (MMP-8 P= 0.03, MMP-9 P = 0.03) and end-systolic volumes at 1 year (MMP-

8 $P = 0.005$, MMP-9 $P = 0.005$) and retained independent predictive value when adjusting on BNP. Baseline serum MMP-8 was the only significant predictor of cardiovascular outcome ($P= 0.03$). Taking into account both MMP-8 and BNP further improved risk stratification. Patients with high MMP-8 level (≥ 27 ng/ml) and high BNP level (≥130 pg/ml) had a significant higher risk ($p<0.0001$). Baseline serum MMP-8 is a significant predictor of LV remodelling and cardiovascular outcomes in post-MI and may help to improve risk stratification.

Thus the present invention relates to a method for predicting the survival time of a post acute myocardial infarction patient comprising i) determining the level of MMP-8 in a blood sample obtained from the patient, ii) comparing the level determined at step i) with a predetermined reference value and iii) providing a bad prognosis when the level determined at step i) is higher than the predetermined reference level and a good prognosis when the level determined at step i) is lower than the predetermined reference value.

As used herein the term "MMP-8" has its general meaning in the art and refers to the metalloproteinase 8.

The predetermined reference value used for comparison may consist of "cut-off" value that may be determined as described hereunder. Each predetermined reference value ("cut-off") value may be determined by carrying out a method comprising the steps of

a) providing a collection of blood samples from post acute myocardial infarction patients;

b) providing, for each blood sample provided at step a), information relating to the actual clinical outcome for the corresponding post acute myocardial infarction patient (i.e. the duration of the disease-free survival (DFS) and/or the overall survival (OS));

c) providing a serial of arbitrary quantification values;

d) determining the level of MMP-8 for each blood sample contained in the collection provided at step a);

e) classifying said blood samples in two groups for one specific arbitrary quantification value provided at step c), respectively: (i) a first group comprising blood samples that exhibit a quantification value for level that is lower than the said arbitrary quantification value contained in the said serial of quantification values; (ii) a second group comprising blood samples that exhibit a quantification value for said level that is higher than

the said arbitrary quantification value contained in the said serial of quantification values; whereby two groups of blood samples are obtained for the said specific quantification value, wherein the blood samples of each group are separately enumerated;

f) calculating the statistical significance between (i) the quantification value obtained at step e) and (ii) the actual clinical outcome of the patients from which blood samples contained in the first and second groups defined at step f) derive;

g) reiterating steps f) and g) until every arbitrary quantification value provided at step d) is tested;

h) setting the said predetermined reference value ("cut-off" value) as consisting of the arbitrary quantification value for which the highest statistical significance (most significant) has been calculated at step g).

As it is disclosed above, this method allows the setting of a single "cut-off" value that permits discrimination between a poor and a good prognosis with respect to DFS and OS. Practically, high statistical significance values (e.g. low P values) are generally obtained for a range of successive arbitrary quantification values, and not only for a single arbitrary quantification value. Thus, in one alternative embodiment of the method of determining "cut-off" values as above, a minimal statistical significance value (minimal threshold of significance, e.g. maximal threshold P value) is arbitrarily set and a range of a plurality of arbitrary quantification values for which the statistical significance value calculated at step g) is higher (more significant, e.g. lower P value) are retained, so that a range of quantification values is provided. This range of quantification values includes a "cut-off" value as described above. According to this specific embodiment of a "cut-off" value, poor or good clinical outcome prognosis can be determined by comparing the level of MMP-8 determined at step d) with the range of values which are identified. In certain embodiments, a cut-off value thus consists of a range of quantification values, e.g. centered on the quantification value for which the highest statistical significance value is found (e.g. generally the minimum P value which is found). For example, on a hypothetical scale of 1 to 10, if the ideal cut-off value (the value with the highest statistical significance) is 5, a suitable (exemplary) range may be from 4-6. Therefore, a patient may be assessed by comparing values obtained by measuring the level of MMP-8, where values greater than 5 indicate a poor prognosis and values less than 5 indicate a good prognosis; or a patient may be assessed by comparing values obtained by measuring the level of MMP-8 and comparing the values on a scale, where values above the range of 4-6 indicate a poor prognosis and values below the range of 4-6 indicate a good prognosis, with values falling within the range of 4-6 indicating an intermediate prognosis.

In a particular embodiment the method of the invention comprises comparison steps which include a classification of the quantification values measured for the level of MMP-8 in two groups, respectively: (i) a first group termed "Hi" when the quantification value for the level of MMP-8 is higher than the predetermined corresponding reference value and (ii) a second group termed "Lo" when the quantification value for the level of MMP-8 is lower than the predetermined corresponding reference value. Accordingly if the result of the comparison step consists of a "Hi" value, then a bad prognosis is provided. Conversely, if the result of the comparison step consists of a "Lo" value, then a good prognosis is provided.

Typically, the predetermined reference value may be 27 ng/ml (see figure 2).

According to the invention, the measure of level of MMP-8 can be performed by a variety of techniques. Typically, the methods may comprise contacting the sample with a binding partner capable of selectively interacting with MMP-8 in the sample. In some aspects, the binding partners are antibodies, such as, for example, monoclonal antibodies or even aptamers as above described.

The aforementioned assays generally involve the binding of the partner (ie. antibody or aptamer) to 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); polystyrene latex (e.g., beads or microtiter plates); polyvinylidine fluoride; diazotized paper; nylon membranes; activated beads, magnetically responsive beads, and the like.

The level of MMP-8 may be measured by using standard immunodiagnostic techniques, including immunoassays such as competition, direct reaction, or sandwich type assays. Such assays include, but are not limited to, agglutination tests; enzyme-labelled and mediated immunoassays, such as ELISAs; biotin/avidin type assays; radioimmunoassays; immunoelectrophoresis; immunoprecipitation.

An exemplary biochemical test for identifying specific proteins employs a standardized test format, such as ELISA test, although the information provided herein may apply to the development of other biochemical or diagnostic tests and is not limited to the development of an ELISA test (see, e.g., Molecular Immunology: A Textbook, edited by Atassi et al. Marcel Dekker Inc., New York and Basel 1984, for a description of ELISA tests). It is understood that commercial assay enzyme-linked immunosorbant assay (ELISA) kits for various plasma constituents are available. Therefore ELISA method can be used, wherein the

wells of a microtiter plate are coated with a set of antibodies which recognize MMP-8. A sample containing or suspected of containing MMP-8 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 labelled 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.

Measuring the level of MMP-8 (with or without immunoassay-based methods) may also include separation of the compounds: centrifugation based on the compound'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 compound's affinity for the particular solid-phase that is used. Once separated, said one or two biomarkers proteins may be identified based on the known "separation profile" e. g., retention time, for that compound and measured using standard techniques.

Alternatively, the separated compounds may be detected and measured by, for example, a mass spectrometer.

Typically, levels of immunoreactive MMP-8 in a sample may be measured by an immunometric assay on the basis of a double-antibody "sandwich" technique, with a monoclonal antibody specific for MMP-8 (Cayman Chemical Company, Ann Arbor, Michigan). Preferably, the antibody has no cross-reactivity with the other types of metalloproteinase such as MMP-9. According to said embodiment, said means for measuring MMP-8 level are for example i) a MMP-8 buffer, ii) a monoclonal antibody that interacts specifically with MMP-8, iii) an enzyme-conjugated antibody specific for MMP-8 and a predetermined reference value of MMP-8.

In one embodiment, the method of the invention may further comprises the steps consisting in a) determining the level of BNP in the blood sample obtained from the patient, b) comparing the level determined at step a) with a predetermined reference value and iii) providing a bad prognosis when the level determined at step a) is higher than the predetermined reference level and a good prognosis when the level determined at step a) is lower than the predetermined reference value.

The predetermined reference value may be determined by the same as described for MMP8. Typically a predetermined reference value for BNP is 130pg/ml (see EXAMPLE).

When the levels determined for MMP8 and for BNP are both higher than their respective predetermined reference values, a poor prognosis is provided. On the contrary when the levels determined for MMP8 and for BNP are both lower than their respective predetermined reference values, a good prognosis is provided. Intermediate prognosis are provided when the levels of MMP8 and BNP are not both higher or lower than their respective predetermined reference values (see Table A).

Table A summary of the prognosis when the levels of MMP8 and BNP are determined.

Score MMP8/BNP	Prognosis
Lo/Lo (levels of MMP8 and BNP are lower than their respective predetermined reference values)	Good Prognosis
Hi/Lo (the level of MMP8 is higher than its predetermined reference value and the level of BNP is lower than its predetermined reference value)	Intermediate prognosis
Lo/Hi (the level of MMP8 is lower than its predetermined reference value and the level of BNP is higher than its predetermined reference value)	Intermediate prognosis
Hi/Hi (levels of MMP8 and BNP are highe than their respective predetermined reference values)	Poor prognosis

In a particular embodiment, the method as described here above is particularly suitable for monitoring the effectiveness of treatment of post acute myocardial infarction. The efficacy of the treatment will be reflected by changes in the measurements of the MMP-8 levels (or the combined values of MMP-8 and BNP levels). Typically, an efficient treatment will enable to get MMP-8 levels that will decrease compared to the levels of MMP-8 measured before the treatment, suggesting that the survival time of the patient will be improved. In another embodiment of the invention, the method as described here above is for selecting a treatment

regimen for a patient diagnosed with a bad prognosis according to the method of the invention.

A further object of the invention relates to a kit for performing the above described method, said kit comprising means for measuring the level of MMP-8 and optionally means for measuring level of BNP in the blood sample obtained from the patient.

In a particular embodiment, said means for measuring the level of MMP-8 is an antibody that interacts specifically with MMP-8. In another embodiment, said means for measuring the level of MMP-8 may be an aptamer or any other binding partner that specifically recognizes MMP-8.

In another embodiment, said kit further comprises means for measuring the level of BNP. In a particular embodiment, said means for measuring the level of BNP is an antibody that interacts specifically with BNP. In another embodiment, said means for measuring the level of BNP may be an aptamer or any other binding partner that specifically recognizes BNP.

Said binding partner(s) can be tagged for an easier detection. It may or may not be immobilized on a substrate surface (e.g., beads, array, and the like). For example, an inventive kit may include an array for predicting the risk of having a cardiovascular event as provided herein. Alternatively, a substrate surface (e.g. membrane) may be included in an inventive kit for immobilization of the binding partner (e.g., via gel electrophoresis and transfer to membrane).

In addition, a kit of the invention generally also comprises at least one reagent for the detection of a complex between binding partner included in the kit and biomarker of the invention.

Depending on the procedure, the kit may further comprise one or more of: extraction buffer and/or reagents, western blotting buffer and/or reagents, and detection means. Protocols for using these buffers and reagents for performing different steps of the procedure may be included in the kit.

The different reagents included in a kit of the invention may be supplied in a solid (e.g. lyophilized) or liquid form. The kits of the present invention may optionally comprise different containers (e.g., vial, ampoule, test tube, flask or bottle) for each individual buffer and/or reagent. Each component will generally be suitable as aliquoted in its respective container or provided in a concentrated form. Other containers suitable for conducting certain

steps of the disclosed methods may also be provided. The individual containers of the kit are preferably maintained in close confinement for commercial sale.

In certain embodiments, a kit comprises instructions for using its components for the prediction of a cardiovascular event in a patient according to a method of the invention. Instructions for using the kit according to methods of the invention may comprise instructions for processing the biological sample obtained from the patient and/or for performing the test, or instructions for interpreting the results. A kit may also contain a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products.

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. LV volumes at 1 year according to tertiles of serum MMP-8 (A) and serum (B) MMP-9 at baseline. Tertile 1 corresponds to low levels of MMPs; Tertile 2, to intermediate levels and Tertile 3, to high levels. Values are mean ± SEM. P values are for linear regression between MMP levels and the variables of interest.

Figure 2. Kaplan–Meier survival curves for the composite endpoint (death or heart failure) according to baseline MMP-8 level (A) and according to baseline MMP-8 and BNP levels (B).

EXAMPLE:

Material & Methods

Study population

The design, and inclusion and exclusion criteria of the REVE-2 study have been published in detail elsewhere[4]. REVE-2 was a multicenter and prospective study designed to analyze the association of circulating biomarkers with LV remodelling. We enrolled 246 patients with a first anterior wall Q-wave MI between February 2006 and September 2008.

Inclusion criteria were hospitalization within 24 hours after symptom onset, and at least 3 LV segments of the infarct zone that were akinetic at the predischarge echocardiography. Exclusion criteria were inadequate echographic image quality, life-limiting noncardiac disease, significant valvular disease, or prior Q-wave MI. The research protocol was approved by the Ethics Committee of the Centre Hospitalier et Universitaire de Lille, and written informed consent was obtained from each patient. The protocol required serial echographic studies at hospital discharge (day 3 to day 7), and 3 months and 1 year after MI; serial blood sampling was performed at hospital discharge (day 3 to day 7), and 1 month, 3 months, and 1 year after MI. Clinical follow-up was achieved at 3 years by contacting patient's cardiologist or general practitioner. The endpoint was a composite of cardiovascular death or hospitalization for heart failure episode during 3 years follow-up.

Echocardiographic studies

A standard echographic imaging protocol was used based on apical 4- and 2-chamber views; 2D echocardiograms of the LV short axis were recorded from the left parasternal region at 3 levels: the mitral valve, mid-papillary muscle, and apex. All echocardiograms were analyzed at the Lille Core Echo Laboratory (Lille, France), as previously described[3]. Left ventricular volumes and ejection fraction (EF) were calculated using a modified Simpson's rule.

Measurements of circulating biomarkers

For each patient, serum and plasma (ethylenediaminetetraacetic acid (EDTA) used as anticoagulant) were collected in glass tubes at the 4 time points indicated above. Serum and plasma were processed within 2 h, and samples were divided into aliquots and stored at -80°C. Samples underwent no more than two freeze/thaw cycles before analysis in a core laboratory (Lille, France). MMPs and TIMPs were measured in serum samples. Serum levels of MMP-1, -2, -3, -8, -9 and -13 were measured using a multiplex luminex kit for simultaneous quantitative detection of human MMPs, according to the manufacturer's instructions (Human MMP Panel Fluorokine Multi Analyte Profiling (MAP) Kit, R&D Systems, Minneapolis, Minneapolis). Serum samples required a 10-fold dilution for MMP-1, -2, -3, -8 and -13 measurement and a 100-fold dilution for MMP-9 according to the protocol. MMP-13 was not detected in our samples (concentration was below detection limit of 0.71 ng/ml). Serum levels of TIMP-1, -2, -3 and -4 were measured using a multiplex tissue inhibitors of metalloproteinases immunoassay (Human TIMP Fluorokine MAP 4-plex Kit,

R&D Systems), samples requiring a 50-fold dilution. TIMP-3 was quantified in one third of samples (detection limit at 7.75 ng/ml). Baseline MMP-9 level was also measured in plasma samples in all patients using the Milliplex MAP Human MMP Panel 2 (Millipore Corp, Billerica, MA), plasma samples requiring a 20-fold dilution. To assess reproducibility of our data, we compared baseline serum MMP-9 level measured by kit from R&D Systems or Millipore in a subgroup of 60 patients. All the samples were analyzed using the Bio-Plex system (Bio-Rad Laboratories, Hercules, CA) according to the instructions from the manufacturer. Analysis of experimental data was performed using four-parameter logistic curve fitting to the standard analyte curves. Using Human MMP Panel Fluorokine MAP Kit and Human TIMP Fluorokine MAP 4-plex Kit, the intraassay coefficients of variation (CV) were < 10% for serum MMPs and TIMPs; mean interassay CV was 9% for MMPs and 16% for TIMPs. Using Milliplex MAP Human MMP Panel 2, intra- and interassay CV were in plasma, 6% and 11% respectively, and, in serum, 8% and 9.7%, respectively. B-type natriuretic peptide (BNP) was measured in plasma samples using a fully automated 2-sites sandwich immunoassay on an Advia Centaur (Siemens Diagnostic, Zurich, Switzerland). The lowest concentration measurable with this assay with a $\leq$ 20% CV is 2.5 pg/mL. The precision of this technique is 2.3% to 4.7%.

Statistical analysis

Results are presented as the mean ± SD, median with 25^{th} and 75^{th} percentiles, or frequency expressed as a percentage. Variables with skewed distribution were log-transformed before being used as continuous variables in statistical analyses. Continuous variables were compared with the Student's *t*-test or with simple linear regression, as appropriate. Discrete variables were compared using χ^2 analysis. Changes in circulating biomarkers over time were assessed by repeated measures ANOVA with post hoc test of Scheffe. Association of MMPs and TIMPs with LV remodelling was studied by linear regression analysis. To adjust for multiple testing, we used the Benjamini & Hochberg procedure to control false discovery rate[13]. MMPs with significant univariate association were entered into a linear regression model with BNP for the prediction of LV remodelling. Cox proportional hazard analysis was performed to determine predictors of cardiac events free survival. We estimated the strength of association with outcome using individual concentrations of BNP and MMP-8 identified from receiver operator characteristic (ROC) curves of these biomarkers. These cut-off points were identified as the point on the curve showing the highest combination of sensitivity and specificity and used to assess the strength

of association with adverse outcome using Kaplan–Meier assessment. Differences in event free survival were compared with a log-rank test. A p value <0.05 was considered statistically significant. SPSS software version 13 (SPPS, Chicago, Illinois) was used for the statistical analysis.

Results

Clinical and echocardiographic characteristics

Baseline and follow-up data on the REVE-2 cohort have been published previously[4]. Briefly, most patients were men (mean age of 57±14 years). In most cases, the index MI was the first manifestation of coronary artery disease. Initial reperfusion therapy was primary percutaneous coronary intervention (PCI) in 128 patients and thrombolysis in 87 patients. Nearly all patients received secondary preventive treatment (beta-blockers 97%, angiotensin-converting enzyme inhibitors 97%, statins 94%, antiplatelet therapy 100%). Echocardiographic follow-up was achieved in 226 of the 242 eligible patients (93%). In the overall cohort, LV remodelling was documented by an increase in end-diastolic volume (EDV) (baseline 52.3±14.0 mL/m^2, 1 year 62.3±18.4 mL/m^2, P <0.0001) and in end-systolic volume (ESV) (baseline 26.8±10.5 mL/m^2, 1 year 29.0±14.5 mL/m^2, P <0.001) at 1-year follow-up. Clinical follow-up data were obtained for 245 patients at a median of 1098 days: 16 patients were hospitalized for heart failure and 15 patients died (11 from cardiac causes).

Temporal profile of MMPs/TIMPs

Serum levels of MMP-1, -2, -3, -8, -9, and TIMP-1, -2, -4 obtained at baseline, 1 month, 3 months, and 1 year after MI are summarized in Table 1. Overall, 3 temporal patterns were identified: MMP-1 level remained stable throughout the study; MMP-2, MMP-3, TIMP-2, and TIMP-4 were significantly lower on the baseline sample and increased thereafter; by contrast, MMP-8, MMP-9 and TIMP-1 were significantly higher at baseline and decreased in the chronic phase.

The simultaneous measurement of several different MMPs and all TIMPs enabled us to investigate potentially significant interrelationships: TIMP-2 showed significant positive correlation with MMP-2, TIMP-1 and TIMP-4. The highest correlation has been observed between baseline MMP-8 and MMP-9 (Table 2) and was also found at 1 month, 3 months and 1 year (respectively r = 0.802, r = 0.775, r = 0.816). We also investigated the relationship between circulating levels of MMPs and TIMPs and several traditional cardiovascular risk

factors or peak creatine kinase (CK), as shown in Table online. Serum levels of MMP-2, MMP-3, TIMP-1, TIMP-2 and TIMP-4 were significantly associated with age of patients. Serum levels of MMP-2 and TIMP-4 were significantly higher in women than men, in contrast with MMP-3. Elevated serum levels of MMP-3 and TIMP-4 were associated with a higher incidence of hypertension. We also found that elevated serum levels of MMP-3, TIMP-2 and TIMP-4 were associated with presence of diabetes. By contrast, serum levels of MMP-1, MMP-8 and MMP-9 were not associated with any baseline characteristics of the study population. Of note, there was no association between any MMPs/TIMPs and infarct size as indicated by the peak CK.

Associations with LVR

Associations of levels of MMPs and TIMPs at baseline and during follow-up with echocardiographic markers of LV remodelling at 1-year follow-up are shown in Table 3. Baseline serum levels of MMP-8 and MMP-9 were associated with LV volumes (EDV and ESV) at 1 year showing positive significant relationships. When assessed at 1 month, 3 months or 1 year, MMP-8 and MMP-9 were not associated with LV remodelling. MMP-1, -2, -3, TIMP-1, -2 and -4 when assessed at baseline and during 1-year follow-up were not associated with LV volumes at 1 year. Figure 1 illustrates the predictive value of baseline serum MMP-8 and MMP-9 by showing LV volumes at 1 year according to tertiles of MMP-8 and MMP-9, respectively. We entered BNP with MMP-8 or MMP-9 into models of prediction of LV remodelling. Two different models (model 1 and model 2) were constructed due to the high correlation between MMP-8 and MMP-9. In each model, only two biological factors were included: the MMP of interest and BNP (Table 4). When adjusting on BNP, baseline serum MMP-8 and MMP-9 retained respectively independent predictive value for LV volumes at 1 year.

Type of sample, plasma or serum, is known to affect measured concentrations of circulating MMP-9. As in most studies MMP-9 was measured in plasma, to strengthen our data, we also measured MMP-9 in plasma of 246 patients in REVE 2 study. Baseline plasma MMP-9 concentration was lower than in serum with a median value of 33 [19-54] ng/ml compared to 517 [360-801] ng/ml in serum. Due to the change of manufacturer for plasma MMP-9, we tested correlation between the kits used by comparing paired serum MMP-9 levels at baseline in a subgroup of 60 patients and found a good correlation ($r=0.93$). Baseline plasma MMP-9 level was weakly associated with ESV at 1 year ($r = 0.180$, $p = 0.027$) but was not associated with EDV at 1 year ($r = 0.131$, $p=0.135$).

Clinical endpoint and prediction of outcome

Baseline MMP-8 was the only significant predictor of cardiovascular outcome (Table 5). When measured at 1 month, 3 months or 1 year, none of the MMPs or TIMPs were associated with outcome. From ROC curves we identified concentrations of baseline serum MMP-8 (27 ng/ml) having the best combination of sensitivity and specificity for prediction of clinical outcome. Patients with MMP-8 $\geq$ 27 ng/ml had markedly increased risk of cardiovascular death or hospitalization for heart failure (Hazard ratio (HR) = 5.45, 95% CI 2.11-14.05, log rank p<0.0001) (Figure 2A). When BNP was entered in the model, both BNP and MMP-8 remained independent predictors of outcome (BNP, HR = 3.16, 95% CI 1.14-8.76, p = 0.03; MMP-8, HR = 4.43, 95% CI 1.70-11.60, p=0.002). Taking into account both BNP and MMP-8 further improved risk stratification. Patients with high MMP-8 level ($\geq$ 27 ng/ml) and high BNP level ($\geq$130 pg/ml) had a significant higher risk (HR=7.52, 95% CI 3.11-18.16, p<0.0001) than patients with low MMP-8 levels or high MMP-8 level but low BNP level (Figure 2B).

Discussion:

The present study is the first to provide an in depth investigation of the temporal pattern of many circulating MMPs and all TIMPs during one-year follow-up after acute MI and their relation with LV remodelling and prognosis in a large cohort of patients. Compared to previous investigations, our study has clear strengths related to its prospective design: relatively high number of patients, very homogeneous study population with a first anterior ST-elevation MI, high rate of reperfusion, nearly systematic use of secondary prevention treatments, serial blood sampling at pre-specified time points during one-year follow-up, prospective assessment of one-year LV remodelling with core lab analysis. Another strength is related to the availability of bead-based multi-analyte profiling technology that can multiplex many MMPs or all TIMPs in a single sample and allowed to study a large panel of biomarkers of ECM turnover.

Changes in circulating MMPs/TIMPs levels during the post-MI period

A large number of studies, performed in animal models of MI, have documented selective and time-dependent modulation of MMPs and TIMPs in the heart during the post-MI period[8]. In humans, however, myocardial measurements of MMPs and TIMPs in the

context of MI are much more difficult. Therefore, the monitoring of MMPs/TIMPs levels/activity in the peripheral blood has emerged as an alternative strategy. Several studies have indeed demonstrated that changes in circulating MMPs/TIMPs levels may be predictive of changes occurring within the myocardium[14]. It must however be acknowledged that the concentrations of MMPs/TIMPs in the peripheral blood is at best an indicator of the localized process that is occurring within the myocardium and that, due to different interactions, certain MMPs or TIMPs may not egress into the circulation[8].

Our study, investigating many MMPs and TIMPs by serial blood sampling during one-year follow-up in 246 patients, is the most extensive description of the temporal pattern of MMPs and TIMPs during the post-MI period. Specifically, early peaks in MMP-8, MMP-9 and TIMP-1 with subsequent decrease during the chronic stage and decreased levels of MMP-2, TIMP-2 and TIMP-4 in the early period occurred after MI: these data are in agreement with the study of Webb *et al.*[15]. Finally, we found decreased levels of MMP-3 in acute phase of MI, as previously described by Samnegard *et al.*[16]. The present study, which was performed in a much larger study population, therefore reinforces the concept of a specific temporal pattern of MMPs and TIMPs release after MI, probably reflecting a local shift in cell type activation as well ECM proteolytic events.

Preanalytical conditions are known to affect measured concentrations of circulating MMPs and TIMPs, including the type of sample (plasma or serum), the anticoagulant used to collect plasma and the freeze-thaw cycles. In this study, we have found higher MMP-9 concentrations in serum compared with plasma, in agreement with previous studies[17]. The observed difference could be attributed to the different release of this analyte from blood cells during platelet activation or sampling process.

Our study, with a precise definition of phenotype, confirms previous findings of associations of serum MMPs and TIMPs with cardiovascular risk factors. In particular, we found positive associations with age for MMP-2, -3 and TIMPs and no associations for MMP-1, -8 and -9, confirming in a larger population the results of Bonnema *et al.*[18]. Moreover, our results are in agreement with the largest study of Gaubatz *et al.*[19], except for MMP-1 and -8. We also confirmed that MMP-3 was significantly increased in men[19,20] and higher in hypertensive and diabetics patients[16]. Finally, our study is the first to describe the association of TIMP-2 and -4 with cardiovascular risk factors in a large population: we found TIMP-2 and -4 were significantly higher in diabetics patients and TIMP-4 in hypertensive patients. Our findings show that circulating levels of MMPs and TIMPs could be associated with an adverse cardiovascular risk profile.

We also investigated the interrelationships among MMPs and TIMPs concentrations and found that levels of MMP-8 and -9 were highly correlated with each another, in agreement with previous studies[19,21,22]. MMP-8 and -9 are both synthesized by differentiating granulocytes, stored in circulating neutrophils, and released after neutrophil activation, a mutuality that could partially explain their association.

Associations of MMPs/TIMPs levels with post-MI outcome

The present study establishes for the first time a positive association between baseline serum MMP-8 and the extent of LV remodelling after MI. To date, only one study was reported[7], finding no association with LV remodelling, probably due to a limited number of patients[15]. Our data are in agreement with experimental studies suggesting that increased MMP-8 activity in the infarct area, caused by a more prominent infiltration of inflammatory cells, contribute to infarct rupture in humans[23]. Regarding MMP-9, our study confirms in serum previous findings of positive relationships of plasma MMP-9 with LV remodelling[15,24-26]. Size of these studies was however often limited with only one study including > 100 patients[25]. Our study, including 246 patients, confirms a positive association between MMP-9 and LV remodelling, making of MMP-9 one of the most consistently biomarker associated with LV remodelling. In our study, serum MMP-9 is a better predictor of LV remodelling than plasma MMP-9. These data suggest that the early expression of MMP-8 and MMP-9 may indicate the onset of intramyocardial processes, which will ultimately lead to LV dilation and dysfunction. Finally, the demonstration that early MMP-8 or MMP-9 levels retain their predictive value when BNP is taken into account could be explained by the fact that these biological markers provide independent information, on extracellular matrix turnover and pressure overload, respectively, and may be used together for risk stratification.

Our study found no strong evidence for association of baseline MMP-1, -2, -3, and TIMP-1, -2, -4 with LV remodelling. Our data are consistent with several earlier studies for MMP-2[15,24,27-29] but differ for MMP-3, with two previous studies showing positive associations[20,29]. Regarding TIMP-1, -2 and -4, the review of the published evidence of relationship with LV remodelling has shown discordant results[7]. The same group found that TIMP-1 concentration correlated with LV volumes and remodelling in a cohort of 404 MI patients[25] but not in a separate cohort of 100 MI patients[30]. Such discrepancies could be explained by methodological differences between studies.

As LV remodelling is associated with increased risk of death and heart failure in post-MI, we investigated associations of MMPs and TIMPs with cardiovascular outcome. MMP-8

was identified as a strong predictor of prognosis after MI, with similar predictive value to that of BNP, a peptide with powerful association with outcome after MI[31]. We have even demonstrated the additive prognostic value of considering both MMP-8 and BNP. To date only a few studies have investigated the association of MMP-8 with cardiovascular outcome. In the study of Tuomainen *et al.*[32], higher serum MMP-8 was associated with the worst cardiovascular outcome in patients with atherosclerosis, possibly by involvement of MMP-8 in vascular matrix remodelling and rupture of unstable plaques[33]. Our study is the first to show serum MMP-8 is an independent predictor of outcome in post-MI patients. As MMP-8 is significantly associated with LV remodelling, which is known to be a powerful predictor of cardiovascular events in post-MI patients[2], it suggests that the worst cardiovascular outcome could be explained by increased ECM myocardial remodelling.

Conclusion:

Our data on MMPs/TIMPs reinforce current knowledge on the implication of this system in LV remodelling and heart failure. Although this will have to be validated in large series of patients with clinical follow-up, our study suggests that the early determination of serum MMP-8 and MMP-9 levels may help to detect the patients at risk of LV remodelling. In addition, early MMP-8 was shown to be a strong predictor of prognosis in post-MI patients and combined with BNP, this multibiomarker profile provided better predictive model than when BNP was considered as a single entity. It is now clear that no single biomarker is optimal for screening, diagnosis and potentially disease management. An early identification of the patients prone to LV remodelling and the worst outcome could help to apply a more aggressive therapy for the high-risk group. Finally, it remains to be established whether modifying these changes in circulation MMP/TIMP profiles in the post-MI period may beneficially alter the clinical course.

Table 1. Serum levels of MMPs and TIMPs at baseline and during follow-up

	Timing of blood sampling			
	Baseline (n = 236)	**1 month** (n = 230)	**3 months** (n = 230)	**1 year** (n = 227)
MMP-1	3.8 [2.2-7.2]	4.0 [2.2-6.9]	4.3 [2.3-6.8]	3.8 [2.1-6.6]

MMP-2	176 [155-204]	204 [183-239]	213 [187-241]	212 [191-245]
MMP-3	16.4 [12.0-24.5]	17.9 [13.3-23.0]	17.3 [13.1-25.8]	19.2 [14.3-25.1]
MMP-8	17.5 [9.4-32.0]	12.0 [6.4-21.4]	12.4 [7.5-21.0]	12.0 [5.6-20.2]
MMP-9	517 [360-801]	478 [330-759]	505 [343-762]	476 [289-686]
TIMP-1	153 [127-188]	141 [118-163]	135 [115-163]	131 [110-154]
TIMP-2	81 [71-93]	95 [82-106]	93 [84-110]	91 [83-107]
TIMP-4	1.20 [0.97-1.62]	1.34 [1.08-1.89]	1.37 [1.08-1.83]	1.42 [1.07-1.88]

Concentrations are expressed in ng/mL. Results are presented as the median with 25th and 75th percentiles.

Table 2. Pearson correlation coefficients between baseline circulating levels of different MMPs and TIMPs .

Variable	**MMP-2**	**MMP-3**	**MMP-8**	**MMP-9**	**TIMP-1**	**TIMP-2**	**TIMP-4**
MMP-1	0.072	-0.05	0.337	0.335	0.401	0.193	0.150
MMP-2		0.268	0.063	-0.036	0.132	0.514	0.323
MMP-3			0.100	0.096	0.094	0.122	0.123
MMP-8				0.766	0.227	0.176	0.055
MMP-9					0.175	0.151	-0.016
TIMP-1						0.604	0.391
TIMP-2							0.505
TIMP-4							

Table 3. Regression coefficients for association of MMPs/TIMPs at baseline and during follow-up with echographic markers of LV remodelling 1 year after MI.

	1-year EDV	**1-year ESV**
Baseline MMPs / TIMPs		
MMP-1	0.090	0.086
MMP-2	0.060	0.090

MMP-3	0.031	0.060
MMP-8	0.192*	0.234†
MMP-9	0.188*	0.232†
TIMP-1	0.055	0.098
TIMP-2	0.039	0.049
TIMP-4	-0.066	-0.043
1 month MMPs / TIMPs		
MMP-1	0.106	0.078
MMP-2	-0.004	0.013
MMP-3	-0.044	-0.022
MMP-8	0.103	0.126
MMP-9	0.123	0.107
TIMP-1	-0.038	0.010
TIMP-2	-0.021	-0.03
TIMP-4	-0.058	-0.049
3 months MMPs/TIMPs		
MMP-1	0.123	0.126
MMP-2	0.098	0.079
MMP-3	0.009	0.041
MMP-8	0.040	0.075
MMP-9	-0.001	0.037
TIMP-1	-0.066	-0.033
TIMP-2	-0.036	-0.05
TIMP-4	-0.048	-0.035
1 year MMPs/TIMPs		
MMP-1	0.081	0.081
MMP-2	0.050	0.066
MMP-3	-0.040	-0.026
MMP-8	0.077	0.08
MMP-9	0.049	0.062

TIMP-1	0.037	0.055
TIMP-2	0.062	0.04
TIMP-4	-0.012	-0.017

*, P =0.03; † P=0.005. EDV, end-diastolic volume; ESV, end-systolic volume.

Table 4. Independent biological predictors at baseline of LV remodelling at 1 year.

Dependent variable (1 year)		**Independent variables (baseline)**	**Standardized ß coefficient**	***P***
EDV	*Model 1*	BNP	0.255	0.001
		MMP-8	0.144	0.03
	Model 2	BNP	0.272	< 0.0001
		MMP-9	0.170	0.01
ESV	*Model 1*	BNP	0.263	< 0.0001
		MMP-8	0.184	0.006
	Model 2	BNP	0.285	<0.0001
		MMP-9	0.213	0.001

Table 5. Predictors of cardiac survival.

	Wald Chi-Square	**P Value**
MMP-1	1.64	ns
MMP-2	4.26	ns
MMP-3	0.12	ns
MMP-8	8.93	0.03
MMP-9 (serum)	4.29	ns
MMP-9 (plasma)	0.38	ns
TIMP-1	2.05	ns
TIMP-2	0.002	ns
TIMP-4	1.76	ns

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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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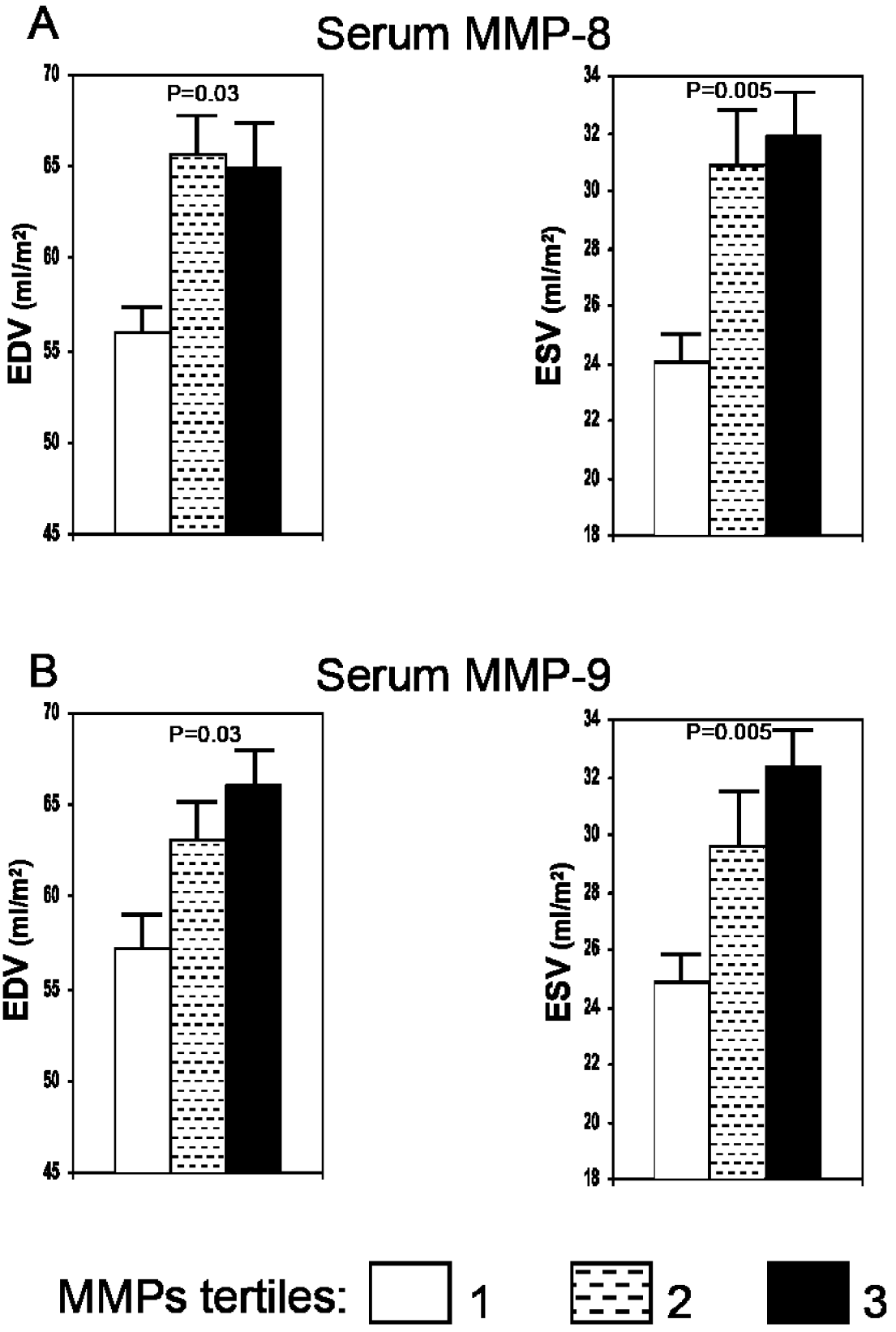
CLAIMS:

1. A method for predicting the survival time of a post acute myocardial infarction patient comprising i) determining the level of MMP-8 in a blood sample obtained from the patient, ii) comparing the level determined at step i) with a predetermined reference value and iii) providing a bad prognosis when the level determined at step i) is higher than the predetermined reference level and a good prognosis when the level determined at step i) is lower than the predetermined reference value.

2. The method according to claim 1 which further comprises the steps consisting in a) determining the level of BNP in the blood sample obtained from the patient, b) comparing the level determined at step a) with a predetermined reference value and c) providing a bad prognosis when the level determined at step a) is higher than the predetermined reference level and a good prognosis when the level determined at step a) is lower than the predetermined reference value.

3. A kit for implementing the method according to claim 2 comprising means for determining the level of MMP-8 and means for determining the level of BNP in a blood sample.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70
65
60
55
50
45
P=0.005
ESV (ml/m²)
34
32
30
28
26
24
22
20
18
B
Serum MMP-9
P=0.03
EDV (ml/m²)
70
65
60
55
50
45
P=0.005
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34
32
30
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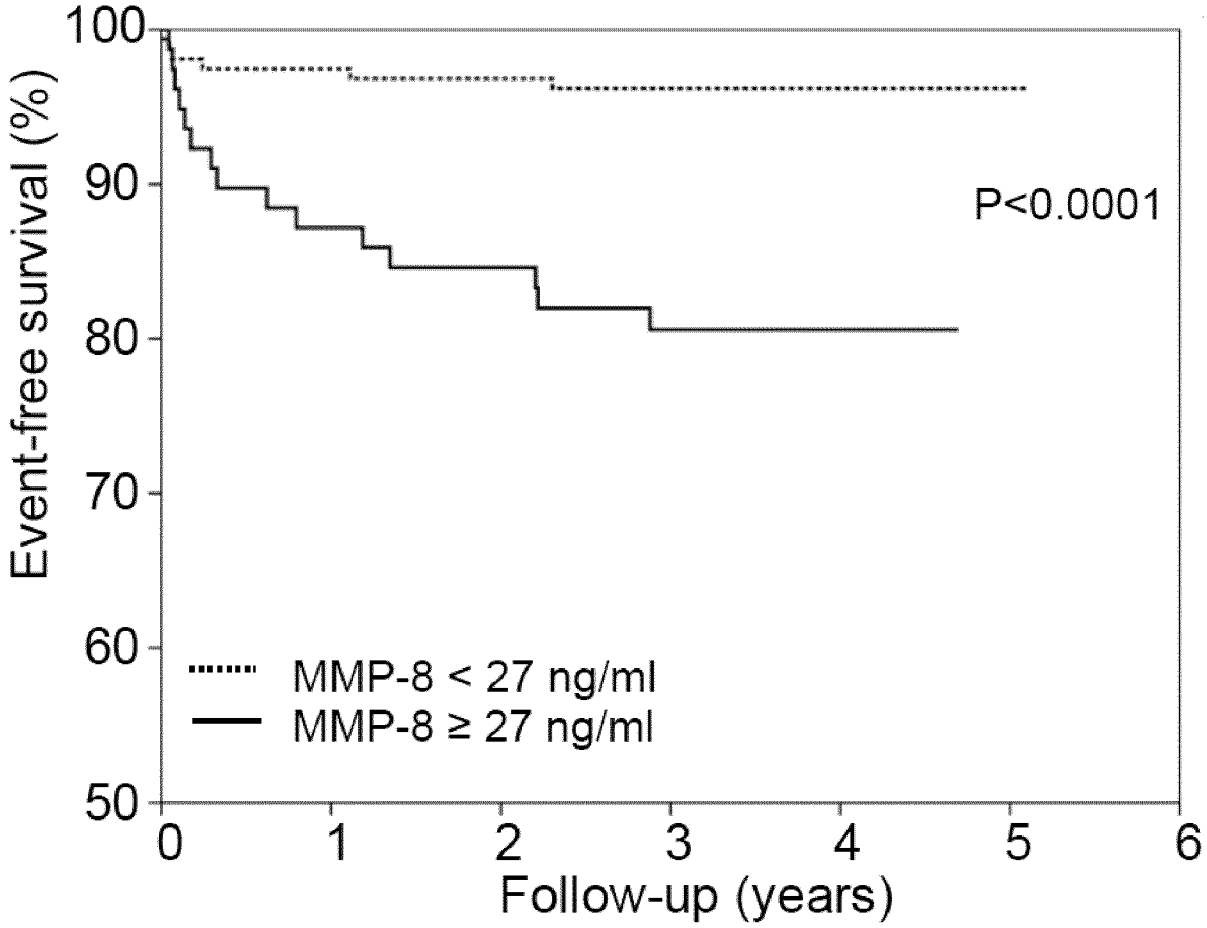
20
18
MMPs tertiles:
1
2
3

Figure 1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80
70
60
50
Event-free survival (%)
P<0.0001
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MMP-8 ≥ 27 ng/ml
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1
2
3
4
5
6
Follow-up (years)

Figure 2A

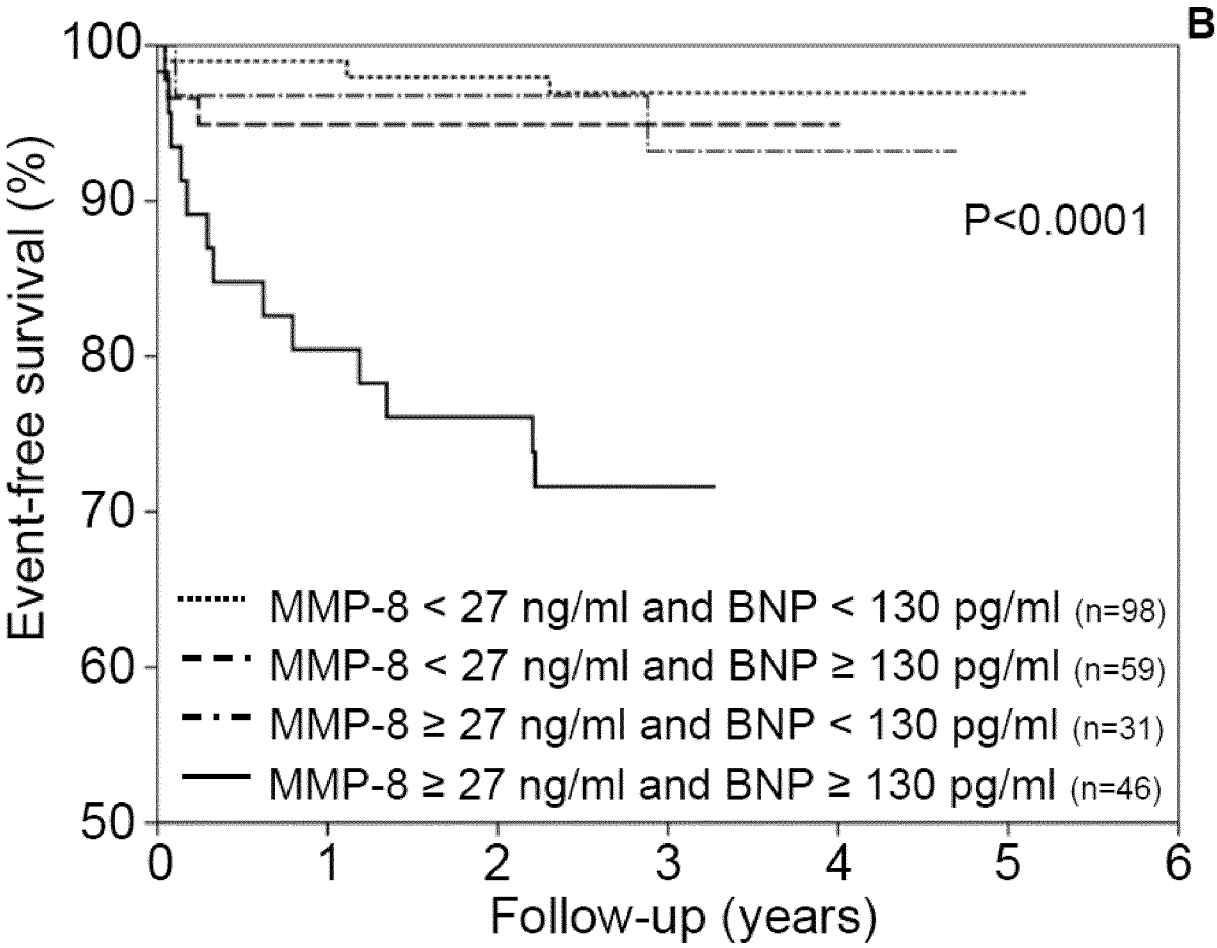
B
100
90
80
70
60
50
Event-free survival (%)
P<0.0001
MMP-8 < 27 ng/ml and BNP < 130 pg/ml (n=98)
MMP-8 < 27 ng/ml and BNP ≥ 130 pg/ml (n=59)
MMP-8 ≥ 27 ng/ml and BNP < 130 pg/ml (n=31)
MMP-8 ≥ 27 ng/ml and BNP ≥ 130 pg/ml (n=46)
0
1
2
3
4
5
6
Follow-up (years)

Figure 2B

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/062885

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

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 practicable, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BRIEST, W., ET AL: "Cardiac remodeling in erythropoietin-transgenic mice", CELL PHYSIOL BIOCHEM, vol. 14, no. 4-6, 2004, pages 277-278, XP009171817, abstract -----	3
A	WO 2005/108987 A2 (RIKSHOSPITALET RADIUMHOSPITALE [NO]; JEMTLAND RUNE [NO]; KJEKSHUS JOHN) 17 November 2005 (2005-11-17) abstract, page 2, line 33 - page 3, line 35, figure 2 -----	1,2
	-/--	

[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

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Date of the actual completion of the international search	Date of mailing of the international search report
9 August 2013	20/08/2013

Name and mailing address of the ISA/	Authorized officer
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Information on patent family members

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