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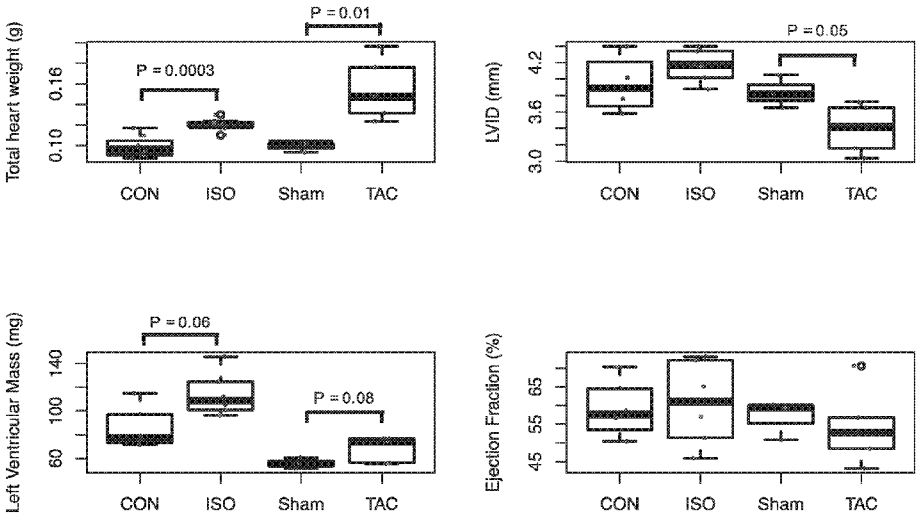

Figure 1

(57) Abstract: Described herein is a method of detecting heart faiiure in a subject. In one embodiment, the method comprises obtaining a biological sample from the subject; and measuring a decreased amount of glycoprotein non-metastatic melanoma protein B (GPNMB) in the sample relative to a reference amount of GPNMB. Optionally, the method further comprises treating the subject for heart failure. Also described is a method of screening for heart failure in a subject, the method comprising: (a) obtaining a biological sample from the subject; (b) measuring the amount of GPNMB in the sample relative to a reference amount of GPNMB; and (c) classifying the subject as having heart failure if the measured amount of GPNMB is decreased relative to the reference amount.

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BIOMARKER FOR PREDICTION OF HEART FAILURE

[0001] This application claims benefit of United States provisional patent application number 62/726,068, filed August 31, 2018, the entire contents of which are incorporated by reference into this application.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0002] This invention was made with government support under Grant Numbers HL123295 and HL129639, awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

[0003] Heart Failure (HF) is a complex disease characterized by a large number of pathological abnormalities including cardiac overload or injury (Braunwald 2008) and the interplay of environmental and genetic factors. B-type natriuretic peptide (BNP) is a hormone produced by the heart. N-terminal (NT)-pro hormone BNP (NT-proBNP) is a non-active prohormone that is released from the same molecule that produces BNP. Both BNP and NT-proBNP are released in response to changes in pressure inside the heart. These changes can be related to heart failure and other cardiac problems. In most cases, BNP and NT-proBNP levels are higher in patients with heart failure than people who have normal heart function.

[0004] There remains a need for more effective and useful markers of HF that are more predictive of HF and that will enable earlier selection of patients for heart transplant and other treatment options.

SUMMARY OF THE INVENTION

[0005] The methods described herein provide a method of detecting heart failure in a subject. In one embodiment, the method comprises obtaining a biological sample from the subject; and measuring a decreased amount of glycoprotein non-metastatic melanoma protein B (GPNMB) in the sample relative to a reference amount of GPNMB. Optionally, the method further comprises treating the subject for heart failure. Also provided is a method of treating heart failure in a subject. In one embodiment, the method comprises obtaining a biological sample from the subject; measuring a decreased amount of GPNMB in the sample relative to a reference amount of GPNMB; and treating the subject for heart failure. In some embodiments, the invention provides a method of screening for heart failure in a subject, the method comprising: (a) obtaining a biological sample from the subject; (b) measuring the

amount of GPNMB in the sample relative to a reference amount of GPNMB; and (c) classifying the subject as having heart failure if the measured amount of GPNMB is decreased relative to the reference amount.

[0006] In some embodiments, the sample comprises plasma, serum, or blood. In some embodiments, the treating comprises administering a heart failure medication, implanting a device, and/or performing a surgical intervention. Representative examples of the medication include, but are not limited to, an angiotensin-converting enzyme (ACE) inhibitor, an angiotensin-II antagonist, a beta-blocker, an If channel blocker, an aldosterone antagonist, a hydralazine and isosorbide dinitrate, and/or a diuretic. In some embodiments, the reference amount of GPNMB is obtained from a healthy, normal control subject.

[0007] In some embodiments, the measuring comprises an immunoassay. One embodiment of the immunoassay is an enzyme-linked immunosorbent assay (ELISA). The invention further provides kits and assay devices for use in the methods described herein.

[0008] Also provided is a method of detecting heart failure in a subject comprising: (a) obtaining an expression product of a gene selected from Serpina3n, AI593442, Gpnmb, Snai3, Spp1, Lox, Gnb3, Catnal1, Retnla, Cdo1, BC020188, BC025833, Angptl7, Pacrg, Arhgdig, Ms4a7, Ccl8, 2310007A19Rik, and Lgals4 in a sample obtained from the subject; (b) measuring the amount of expression product of the gene in the sample relative to a reference amount; and (c) classifying the subject as having heart failure if the measured amount of expression product in the sample is decreased relative to the reference amount. In some embodiments, the method further comprises treating the subject for heart failure. The expression product, in some embodiments, is a nucleotide. In some embodiments, the expression product is a protein.

BRIEF DESCRIPTION OF THE DRAWINGS

[0009] **Figure 1.** Bar graphs showing isoproterenol and transverse aortic constriction induced cardiac remodeling characteristics among C57BL/6J mice. LVID denotes left ventricular internal dimension during diastole. CON denotes control. ISO denotes isoproterenol infusion at 30 mcg/kg/d for 21 days. TAC denotes transverse aortic constriction for 28 days.

[0010] **Figure 2.** GPNMB levels in Isoproterenol, transverse aortic constriction (TAC) heart failure mouse models and in patients with heart failure. A. Western blot analysis of GPNMB expression in C57BL/6J mouse heart lysates after ISO treatment. B. Graphic representation of Western blot analysis for the ISO model. C. GNMB plasma levels comparison between control and ISO-treated mice. For the ISO model, mice were anesthetized with

intraperitoneal ketamine as a surgical anesthetic agent, and osmotic minipumps were implanted subcutaneously, as previously described (Wang, Rau et al. 2016), to deliver isoproterenol (30 mg/kg/day). D. Western blot analysis of GPNMB expression in C57BL/6J mouse heart lysates after TAC surgery. Gapdh used as loading control. * p-value< 0.05 for student's t-test. E. Graphic representation of Western blot analysis for the TAC model. F. GPNMB plasma levels comparison between Sham and TAC. For the TAC model, midsternal incision was made to expose transverse aorta between truncus anonymous and the left carotid artery, as previously described (Sun, Olson et al. 2016). G. GPNMB levels in HF patients and controls from the METSIM study.

[0011] **Figure 3.** Scatterplot showing the correlation between GPNMB, proBNP levels in the METSIM study. The total number of subjects with proBNP and GPNMB levels was n=42.

DETAILED DESCRIPTION OF THE INVENTION

[0012] The invention provides new methods for screening for, detecting, and treating heart failure. These methods are based on the discovery of a marker, GPNMB, that has advantages over known markers of heart failure, as it can be used as an indicator of disease progression and facilitate earlier prediction of heart failure, providing for improved treatment of patients at risk. For example, GPNMB levels in plasma are independent of proBNP levels, suggesting that measurement of GPNMB in plasma of HF patients may provide additional prognostic value or reflect different clinical or biological states from those associated with proBNP elevation. Serum levels of GPNMB decrease with ventricular enlargement, allowing for detection and monitoring of disease progression and identification of patients in need of more aggressive treatment.

Definitions

[0013] All scientific and technical terms used in this application have meanings commonly used in the art unless otherwise specified. As used in this application, the following words or phrases have the meanings specified.

[0014] As used herein, a "control" or "reference" sample means a sample that is representative of normal measures of the respective marker, such as would be obtained from normal, healthy control subjects, or a baseline amount of marker to be used for comparison. The sample can be an actual sample used for testing, or a reference level or range, based on known normal measurements of the corresponding marker.

[0015] As used herein, a "significant difference" means a difference that can be detected in a manner that is considered reliable by one skilled in the art, such as a statistically significant difference, or a difference that is of sufficient magnitude that, under the circumstances, can

be detected with a reasonable level of reliability. In one example, a decrease of 10% relative to a reference sample is a significant difference. In other examples, a decrease of 20%, 30%, 40%, or 50% relative to the reference sample is considered a significant difference.

[0016] As used herein, the term "subject" includes any human or non-human animal. The term "non-human animal" includes all vertebrates, e.g., mammals and non-mammals, such as non-human primates, horses, sheep, dogs, cows, pigs, chickens, and other veterinary subjects. In a typical embodiment, the subject is a human.

[0017] As used herein, "a" or "an" means at least one, unless clearly indicated otherwise.

Methods

[0018] The invention provides methods for screening, detection, prediction, and treatment of heart failure. The methods described herein are particularly useful for predicting the risk of heart failure progression, and can be used to detect and monitor such progression in HF patients.

[0019] In one embodiment, the method comprises obtaining a biological sample from the subject; and measuring a decreased amount of glycoprotein non-metastatic melanoma protein B (GPNMB) in the sample relative to a reference amount of GPNMB. Optionally, the method further comprises treating the subject for heart failure. Also provided is a method of treating heart failure in a subject. In one embodiment, the method comprises obtaining a biological sample from the subject; measuring a decreased amount of GPNMB in the sample relative to a reference amount of GPNMB; and treating the subject for heart failure. In some embodiments, the invention provides a method of screening for heart failure in a subject, the method comprising: (a) obtaining a biological sample from the subject; (b) measuring the amount of GPNMB in the sample relative to a reference amount of GPNMB; and (c) classifying the subject as having heart failure if the measured amount of GPNMB is decreased relative to the reference amount.

[0020] In some embodiments, the treating comprises administering a heart failure medication, implanting a device, and/or performing a surgical intervention. Representative examples of the medication include, but are not limited to, an angiotensin-converting enzyme (ACE) inhibitor, an angiotensin-II antagonist, a beta-blocker, an If channel blocker, an aldosterone antagonist, a hydralazine and isosorbide dinitrate, and/or a diuretic. In some embodiments, the reference amount of GPNMB is obtained from a healthy, normal control subject.

[0021] Also provided is a method of detecting heart failure in a subject comprising: (a) obtaining an expression product of a gene selected from Serpina3n, AI593442, Gpnmb,

Snai3, Spp1, Lox, Gnb3, Catnal1, Retnla, Cdo1, BC020188, BC025833, Angptl7, Pacrg, Arhgdig, Ms4a7, Ccl8, 2310007A19Rik, and Lgals4 in a sample obtained from the subject; (b) measuring the amount of expression product of the gene in the sample relative to a reference amount; and (c) classifying the subject as having heart failure if the measured amount of expression product in the sample is decreased relative to the reference amount. In some embodiments, the method further comprises treating the subject for heart failure.

[0022] In some embodiments of the methods described herein, the sample comprises plasma, serum, or blood. In some embodiments of the methods described herein, the measuring comprises an immunoassay. One embodiment of the immunoassay is an enzyme-linked immunosorbent assay (ELISA). Other representative immunoassays include, but are not limited to, Western blot, immunohistochemistry, immunofluorescence, and competition assays.

[0023] For use in the methods described herein, representative examples of the sample include, but are not limited to, blood, plasma or serum, saliva, urine, cerebral spinal fluid, milk, cervical secretions, semen, tissue, cell cultures, and other bodily fluids or tissue specimens.

Kits and Assay Standards

[0024] The invention provides kits comprising a set of reagents as described herein, such as antibodies that specifically bind one or more markers of the invention (including genes and their expression products), and optionally, one or more suitable containers containing reagents of the invention. Reagents include molecules that specifically bind and/or amplify and/or detect one or more markers of the invention. Such molecules can be provided in the form of a microarray or other article of manufacture for use in an assay described herein. One example of a reagent is an antibody or nucleic acid probe that is specific for the marker(s). Another example includes probes (or primers) that selectively identify one or more genotypes described herein. Reagents can optionally include a detectable label. Labels can be fluorescent, luminescent, enzymatic, chromogenic, or radioactive.

[0025] Kits of the invention optionally comprise an assay standard or a set of assay standards, either separately or together with other reagents. An assay standard can serve as a normal control by providing a reference level of normal expression for a given marker that is representative of a healthy individual.

[0026] Kits can include probes for detection of alternative gene expression products in addition to antibodies for protein detection. The kit can optionally include a buffer. Reagents

and standards can be provided in combinations reflecting the combinations of markers described herein as useful for detection.

EXAMPLES

[0027] The following examples are presented to illustrate the present invention and to assist one of ordinary skill in making and using the same. The examples are not intended in any way to otherwise limit the scope of the invention.

Example 1: Systems Genetics Approach to Biomarker Discovery: GPNMB and Heart Failure in Mice and Humans

[0028] This Example describes a simple bioinformatics method for biomarker discovery that is based on the analysis of global transcript levels in a population of inbred mouse strains showing variation for disease-related traits. This method has advantages such as controlled environment and accessibility to heart and plasma tissue in the preclinical selection stage. We illustrate the approach by identifying candidate heart failure (HF) biomarkers by overlaying mouse transcriptome and clinical traits from 91 Hybrid Mouse Diversity Panel (HMDP) inbred strains and human HF transcriptome from the (Myocardial Applied Genomics Network (MAGNet) consortium. We found that some of the top differentially expressed genes correlated with known human HF biomarkers, such as galectin-3 and tissue inhibitor of metalloproteinase 1. Using ELISA assays, we investigated one novel candidate, Glycoprotein NMB, in a mouse model of chronic β-adrenergic stimulation by isoproterenol (ISO) induced HF. We observed significantly lower GPNMB plasma levels in the ISO model compared to the control group (p-value = 0.007). In addition, we assessed GPNMB plasma levels among 389 HF cases and controls from the METabolic Syndrome In Men (METSIM) study. Lower levels of GPNMB were also observed in patients with HF from the METSIM study compared to non-HF controls (p-value < 0.0001). In summary, we have identified several candidate biomarkers for HF using the cardiac transcriptome data in a population of mice that may be directly relevant and applicable to human populations.

[0029] We have developed a systems genetics resource termed the Hybrid Mouse Diversity Panel (HMDP), where the inbred mice were chosen for diversity. They have been maintained under a variety of environmental conditions, typed for various clinical traits, and subjected to global transcriptomic profiling of relevant tissues (Lusis, Seldin et al. 2016). This Example describes a study for one trait previously investigated in the HMDP, heart failure (HF). This Example shows that the list of genes, whose transcript levels in heart correlate most strongly with HF traits, includes known biomarkers of human HF. Described herein is a novel HF biomarker, Glycoprotein NMB (GPNMB), in both mice and humans. GPNMB is a type 1 transmembrane protein also known as osteoactivin (Selim 2009) that has been

recently involved in inflammation, fibrosis and myocardial remodeling (Jarve, Muhlstedt et al. 2017).

[0030] **Materials and Methods**

[0031] Analysis of Hybrid Mouse Diversity Panel (HMDP) cardiac transcriptome data

[0032] The differential expression of cardiac transcriptome data from 91 inbred strains of the Heart Failure-HMDP study has been published previously (Wang, Rau et al. 2016). We performed correlation analysis of the change in left ventricular internal dimension (LVIDd) from baseline to week 3 of isoproterenol and cardiac transcript levels at week 3 of isoproterenol.

[0033] Analysis of *GPNMB* transcript level in the human Myocardial Applied Genomics Network (MAGNet) study

[0034] In order to confirm the upregulation of *GPNMB* in humans during HF, we examined available human cardiac transcriptome data from the MAGNet consortium. The MAGNet consortium has collected and evaluated the cardiac transcriptome using microarrays for 313 subjects at the time of heart transplant or explant [95 individuals with ischemic cardiomyopathy (ICM), 82 with dilated cardiomyopathy (DCM), and 136 non-heart failure (NF) unused donors (Das, Morley et al. 2015, Liu, Morley et al. 2015). RNA-Seq and microarray data have been deposited in the Gene Expression Omnibus (GEO) Database (Accession number GSE57345). Differential gene expression analysis was performed using GEO2R available on the GEO website.

[0035] Mouse Models of Heart Failure

[0036] We assessed circulating GPNMB levels in 2 well-established mouse HF models: pressure overload by transverse aortic constriction (TAC) and chronic β-adrenergic stimulation by continuous isoproterenol (ISO)-induced cardiac hypertrophy. For the TAC model, mice were divided into TAC or sham surgery groups. Sham mice received a midsternal incision to expose only the transverse aorta. For the ISO model, mice were divided into control and ISO treatment groups. ISO was administered via an intraperitoneal minipump that delivers a continuous infusion of 30 mg/kg/day for 21 days. The ISO dose was determinated according to previously published data and our HMDP study (Oudit, Crackower et al. 2003, Berthonneche, Peter et al. 2009, Galindo, Skinner et al. 2009, Wang, Rau et al. 2016). Both HF models were performed in 10-week-old female C57BL/6J mice.

[0037] Plasma samples were collected by retro-orbital puncture at the time of euthanasia, which was at 4 weeks after intervention for TAC mice (n=6) and at 3 weeks after infusion pump implantation for ISO mice (n=10). Upon conclusion of the experiments, animals were

euthanized and the hearts were removed. We chose C57BL/6J mice to perform our experiments, as it is a standard mouse strain that can be easily compared to prior investigations by other researchers. C57BL/6J showed a moderate level of response to ISO among the HMDP strains and is not a GPNMB-deficient strain such as DBA/2J mice. The UCLA Institutional Animal Care and Use Committee (IACUC) approved all animal studies.

[0038] Echocardiography

[0039] Echocardiograms were performed using the Vevo 2100 ultrasound system (VisualSonics, Inc., Toronto, ON, Canada). A parasternal long-axis B-mode image was obtained. The maximal long-axis of the LV was positioned perpendicular to the ultrasound beam. A 90° rotation of the ultrasound probe at the papillary muscle level was performed to obtain a parasternal short-axis view of the LV. A M-mode image to document LV dimensions was captured and saved for analysis using the Vevo 2100 cardiac analysis package. Baseline echocardiograms were performed on all of the mice. In the isoproterenol cohort, final echocardiograms were performed for control and isoproterenol-treated mice at week 3 of the experiment. In the transaortic constriction cohort, final echocardiograms were performed for control and TAC-treated mice at week 4 of the experiment. To ensure adequate sedation while minimizing the effects of inhaled isoflurane on loading conditions, heart rate, cardiac structure and function, we minimized induction and maintenance doses of isoflurane at or below 1.25% and 1%, respectively, while closely monitoring for HR < 475 bpm as a sign of deep sedation and adjusting isoflurane dosage as needed (Wang, Rau et al. 2016).

[0040] Western blot analysis of GPNMB in heart tissues of mice

[0041] Proteins from the heart tissue of ISO treated, TAC mice, and control mice were harvested in buffer (50mM HEPES [pH7.4], 150mM NaCl, 1% NP-40, 1mM EDTA, 1mM EGTA, 1mM glycerophosphate, 2.5mM sodium pyrophosphate 1mM Na3VO4, 20mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 µg/mL of aprotinin, leupeptin, and pepstatin). Equal amounts of protein were separated on 4-12% Bis-Tris gels (Invitrogen, Carlsbad, CA) using an electroblotting apparatus (Bio-Rad Laboratories, Hercules, CA) and transferred onto a nitrocellulose blot (Amersham, GE Healthcare). The blot was probed with the indicated primary antibodies using the polyclonal anti-GPNMB (R&D Systems, Minneapolis, MN) and anti-GAPDH (Invitrogen, Carlsbad, CA). Protein signals were detected using HRP conjugated secondary antibodies (Cell Signaling Technologies) and enhanced chemiluminescence (ECL) western blotting detection regents (Amersham, GE Healthcare).

[0042] Cross-sectional study of the METabolic Syndrome In Men (METSIM) cohort

[0043] The METSIM study is comprised of 10,197 Finnish men recruited between age 45 to 74 years (mean ± SD = 58 ± 7 years) by random sampling from the population register of Kuopio, Eastern Finland. The METSIM study and its methods have been described in detail elsewhere (Stancakova, Javorsky et al. 2009, Laakso, Kuusisto et al. 2017). The METSIM HF cases were identified by screening medical records for HF diagnostic codes and by querying the Finnish medication reimbursement database for HF medications. A total of 119 subjects with HF were identified and 270 control subjects with no previous diagnosis of HF or current clinical or biochemical indication of cardiovascular diseases or other chronic disease including chronic kidney disease and end stage renal disease patients were determined to be controls. The study was approved by the Ethics Committee of the University of Eastern Finland and Kuopio University Hospital.

[0044] GPNMB measurements in mice and humans

[0045] Plasma GPNMB levels in mice and human samples were assayed using commercial enzyme-linked immunosorbent assay kits (R&D systems, Minneapolis, MN) (Catalogue numbers: DY2330 and DY2550, respectively).

[0046] Statistical analysis

[0047] The t-test statistic was used to examine differences between HF and control plasma GPNMB protein levels in mice. The p-value threshold of < 0.05 was considered statistically significant. Clinical characteristics of HF cases and non-HF controls were compared using t-tests for continuous variables and Fisher's exact tests for categorical variables. A Spearman rank correlation test was used to assess the correlation between GPNMB and proBNP. The associations between GPNMB and HF were investigated by univariate and multivariate logistic regression models using age, BMI, hypertension, diabetes, eGFR and LDL-C levels as covariates to control for potential confounders. These covariates were chosen based on data from previous reports (Wang, Larson et al. 2002, Barasch, Gottdiener et al. 2009, Duprez, Gross et al. 2018) and clinical data available from the METSIM study. All the statistical analyses were performed with the SPSS statistical software package.

[0048] Data availability

[0049] The HMDP cardiac transcriptome data are available at the Gene Expression Omnibus (GEO) online database by the accession GSE48760 (Wang, Rau et al. 2016). The complete correlation data of cardiac transcripts with ISO-induced left ventricular dilation is presented in Supplemental Table 1. Supplemental table 2 includes unidentified clinical data of METSIM HF cases and controls included in this study.

[0050] **Results**

[0051] Selection of GPNMB as a candidate biomarker for HF

[0052] We hypothesized that a plasma biomarker should be robust in terms of fold change in disease versus healthy states; its levels should also correlate with disease severity. Thus, we ranked transcripts that were most perturbed in terms of fold change by isoproterenol (Table 1). We also ranked transcripts by the magnitude of correlation with left ventricular dilation, a clinical trait we used as a surrogate marker of adverse cardiac remodeling (Table 2). Of interest, the top correlated transcripts with left ventricular dilation corresponded to genes involved in collagen synthesis and degradation (*Col6a1 (Luther, Thodeti et al. 2012)*, *Col5a1 (Roulet, Ruggiero et al. 2007)*, *Fbn1 (Fedak, de Sa et al. 2003)*), remodeling of the heart and arterial calcification (*Dtr*, *Spp1 (Peacock, Huk et al. 2011)*, *Enpp1(Pillai, Li et al. 2017)*), extracellular matrix synthesis and degradation (*Ctsk* (Hua, Xu et al. 2013), *Sparc* (Bradshaw 2009, Toba, de Castro Bras et al. 2016) and *Mfap5 (Vaittinen, Kolehmainen et al. 2015)*).

[0053] Table 1. Top differentially regulated genes in the ISO versus control cardiac transcriptome

PROBE_ID	SYMBOL	logFC	AveExpr	p-value
ILMN_3103896	Timp1	2.04	8.75	5.21E-26
ILMN_2769918	Timp1	2.03	8.69	1.08E-25
ILMN_1246800	Serpina3n	2.01	9.15	4.24E-24
ILMN_2654624	AI593442	1.88	8.00	7.90E-28
ILMN_1223317	Lgals3	1.82	8.65	9.73E-26
ILMN_2648669	Gpnmb	1.61	6.97	1.92E-12
ILMN_1239726	Snai3	-1.51	7.03	3.60E-26
ILMN_2690603	Spp1	1.31	5.36	4.40E-11
ILMN_2997494	Lox	1.30	6.91	2.97E-17
ILMN_1218235	Gnb3	-1.19	6.81	1.35E-25
ILMN_1232261	Catnal1	1.18	11.00	7.77E-29
ILMN_1226472	Retnla	-1.17	6.51	6.30E-17
ILMN_2975345	Cdo1	1.17	7.84	5.48E-11
ILMN_3127595	BC020188	1.14	6.55	4.28E-21
ILMN_2666312	BC025833	-1.09	8.77	1.90E-21

ILMN_2844820	Angptl7	1.09	7.80	2.58E-07
ILMN_2625279	Pacrg	1.09	6.78	8.73E-28
ILMN_2950622	Arhgdig	1.07	6.09	2.23E-28
ILMN_3091003	Ms4a7	1.05	7.41	1.14E-22
ILMN_1238886	Ccl8	1.04	6.34	1.82E-11
ILMN_1222196	2310007A19Rik	1.03	6.82	1.20E-23
ILMN_2968211	Lgals4	-1.03	9.78	1.15E-14

[0054] LogFC: Log fold change; AveExpr: Average expression; adj. P Val: adjusted p-value. The Average expression is the ordinary arithmetic average of the log2-expression values for the probe, across all arrays.

[0055] We examined cardiac expression of *Nppb*, *Timp1* and *Lgals3*, which are transcripts encoding three well-known heart failure plasma biomarkers brain natriuretic peptide (BNP), tissue inhibitor of metalloproteinase 1 (TIMP1), and galectin-3 (de Boer, Voors et al. 2009, Goldbergova, Parenica et al. 2012, Ho, Liu et al. 2012). *Timp1* and *Lgals3* increased by 3.5- to 4-fold with isoproterenol treatment (Table 1). Both transcripts were also positively correlated with isoproterenol-induced left ventricular dilation (Table 2; Timp1: r = 0.25, p-value = 0.02; Lgals3: r = 0.29, p-value = 0.006). Interestingly, although *Nppb* level was positively correlated with left ventricular dilation (r = 0.26, p-value = 0.01), *Nppb* level was not significant altered by isoproterenol.

[0056] Table 2. Top correlated transcripts with isoproterenol-induced left ventricular dilation

ilmn_id	symbol	cor	p-value
ILMN_2698449	Dtr	0.433	2.6E-05
ILMN_2768087	Col6a1	0.429	3.1E-05
ILMN_2636424	Itgbl1	0.414	6.2E-05
ILMN_2818294	Srpx2	0.410	7.3E-05
ILMN_2883952	1810015A11Rik	0.410	7.3E-05
ILMN_2887408	Galr3	0.406	8.6E-05
ILMN_2597831	Cacna1c	-0.403	9.9E-05
ILMN_2748402	Col5a1	0.398	1.2E-04
ILMN_2603958	9130427A09Rik	0.391	1.6E-04
ILMN_2721149	Arl11	0.389	1.8E-04
ILMN_2946873	D030070L09Rik	0.388	1.9E-04
ILMN_2638256	Tex16	-0.379	2.7E-04
ILMN_1259388	Col6a1	0.378	2.8E-04
ILMN_2711163	Ctsk	0.378	2.8E-04
ILMN_2811421	Matk	0.377	2.9E-04
ILMN_2782964	Enpp1	0.376	3.1E-04
ILMN_2664660	Aldh5a1	-0.375	3.2E-04
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ILMN_2750201	1700023I07Rik	0.370	3.8E-04
ILMN_2975345	Cdo1	-0.370	3.8E-04
ILMN_2641956	Nab2	-0.370	3.9E-04

ILMN_2833163	BC064033	0.369	4.0E-04
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ILMN_1231851	Enpp1	0.365	4.6E-04
ILMN_2953515	Aldh3b1	0.365	4.7E-04
ILMN_1223552	Fbn1	0.365	4.8E-04
ILMN_2645526	Abcc8	-0.363	5.1E-04
ILMN_2614655	Gpnmb	0.363	5.2E-04
ILMN_1214571	Cd109	0.361	5.5E-04
ILMN_1225835	Mfap5	0.360	5.6E-04
ILMN_2702704	Ndufv1	-0.359	5.9E-04
ILMN_2725484	Padi4	0.359	6.0E-04
ILMN_2691951	Polydom	0.358	6.2E-04

ILMN_1221611	Pitpn	0.357	6.3E-04
ILMN_1228485	Csnk2a2	-0.356	6.6E-04
ILMN_2838317	Pqlc3	0.356	6.6E-04
ILMN_1221800	Gabpa	-0.356	6.6E-04
ILMN_2646254	1700102P08Rik	-0.356	6.6E-04
ILMN_3022719	Wiz	-0.355	6.8E-04
ILMN_2453695	Urod	-0.353	7.5E-04
ILMN_2837100	Gm128	0.352	7.6E-04
ILMN_3116885	Gpr137b	0.352	7.7E-04
ILMN_2487358	Eif3s6	0.351	8.1E-04
ILMN_2671755	Ceecam1	0.351	8.1E-04
ILMN_2492500	Zfhx1a	0.351	8.1E-04

[0057] To determine whether our heart failure model in mice may be relevant to human heart failure, we performed differential gene expression analysis of microarray-based transcriptome data deposited in the Gene Expression Omnibus (GEO) database (GSE57345 GPL9052) from the MAGNet consortium human cardiac tissue collection using GEO2R. MAGNet consortium collected and evaluated the cardiac transcriptome by microarray at the time of heart transplant or explant (Das, Morley et al. 2015, Liu, Morley et al. 2015). *TIMP1* and *LGALS3* were significantly differentially expressed in the MAGNet study (LogFC= -0.69, p-value = 6.32 x10-^{17} and logFC= 0.17, p-value = 8.30 x10-^{6}, respectively). As observed in mice, *NPPB* levels were not differentially expressed between HF cases and control subjects (p-value= 0.32).

[0058] Next, we overlaid the top differentially expressed (Table 1) and correlated (Table 2) lists from the heart failure HMDP to identify novel candidate transcripts that were both differentially regulated by isoproterenol and correlated significantly to left ventricular dilation. *Cdo1* and *Gpnmb* fit both criteria. While Cdo1 was negatively correlated with left ventricular dilation, *Gpnmb* was positively correlated with left ventricular dilation. *Gpnmb* encodes a transmembrane protein expressed in macrophages and has an ectodomain that is shed by its regulatory protein ADAM10 to the extracellular compartment. We chose to follow up on GPNMB after confirming that *GPNMB* transcript levels were similarly upregulated in failing versus non-failing hearts by 1.2-fold (logFC = 0.277 p = 2.9 x 10^{-6}) in subjects from the MAGNet cohort.

[0059] GPNMB levels in two mouse models for HF

[0060] To confirm the protein levels of GPNMB in heart failure, we used two widely accepted modes of cardiac injury, isoproterenol (ISO) and transverse aortic constriction (TAC), to induce a heart failure-like state in mice. Both models lead to cardiac hypertrophy as measured by heart weight at sacrifice and left ventricular mass estimates by echocardiography (Figure 1). Consistent with our observation in the cardiac transcriptome, GPNMB protein expression in the heart was increased in mice treated with ISO as compared to controls (Figure 2A and 2B). Similarly, GPNMB protein level in the TAC hearts also showed a significant increase as compared with sham animals (Figure 2C and 2D), indicating that there is increased GPNMB cardiac expression in two different HF mouse models.

[0061] Furthermore, we measured plasma GPNMB protein levels in the ISO and TAC models. In the ISO model, after 3 weeks of continuous infusion of ISO, plasma levels of GPNMB were lower than in the control group (5.96 ± 2.66 ng/mL in control versus 3.18 ± 1.08 in ISO, p=0.007) (Figure 2C). Although the plasma GPNMB levels in the TAC model compared with the sham surgery group at 4 weeks after surgery were not statistically significantly different due to small sample sizes, there was a trend towards decreased GPNMB levels in the TAC mice (4.19±2.33 ng/mL in Sham versus 2.22±1.80 ng/ml in TAC, p=0.13) (Figure 2F).

[0062] GPNMB levels in human HF from the METSIM study

[0063] Given the unexpected findings replicated in two different heart failure models in mice, we measured plasma GPNMB levels in 119 HF subjects and 270 non-HF controls from the METSIM study. Patients' baseline characteristics are listed in Supplemental Table 3. The distribution of plasma GPNMB did not reveal normality in both control and HF groups, thus we used log GPNMB in these analyses. As observed in the ISO mice, there were significantly lower plasma GPNMB levels in patients with HF compared with non-HF controls (GPNMB 1.20±0.26 ng/mL in control versus 0.74±0.40 ng/mL in heart failure, p<0.0001) (Figure 2G). To prevent bias due to an age difference between HF cases and controls, we performed sensitivity analysis that confirmed our results were not affected by age differences between the groups (p<0.001). GPNMB, age, BMI, history of HTN and DM, eGFR and LDL-C were significantly associated with HF (Table 3) and were included in the multivariate analysis. The association between GPNMB and HF remained significant in the multivariate analyses (OR=0.86 [0.82-0.90], p<0.001). In a subset of HF cases, where proBNP levels were available, GPNMB and proBNP were found to be independent (r=0.028, p-value=0.863), suggesting that measurement of GPNMB in plasma of HF patients may

provide additional prognostic value or reflect different clinical or biological states from those associated with proBNP elevation (Figure 3).

[0064] Table 3. Univariate and multivariate logistic regression analysis of the variables associated with the presence of heart failure

	Univariate analysis		Multivariate analysis	
Variables	OR (95% CI)	*P-value*	OR (95% CI)	*P-value*
GPNMB, ng/Ml	0.865 (0.834-0.896)	<0.001	0.863 (0.824-0.904)	<0.001
Age, years	1.306 (1.233-1.384)	<0.001	1.277 (1.182-1.379)	<0.001
Body mass index kg/m^2	1.188 (1.124-1.256)	<0.001	1.142 (1.057-1.233)	0.001
Hypertension	6.173 (3.703-10.309)	<0.001	2.922 (1.286-6.643)	0.010
Diabetes mellitus	13.699 (6.536-28.571)	<0.001	6.711 (2.128-21.277)	0.001
eGFR, mL/min/1.73 m^2	0.975 (0.960-0.989)	0.001	0.994 (0.971-1.017)	0.603
LDL-c, mg/Dl	0.972 (0.965-0.980)	<0.001	0.991 (0.980-1.002)	0.097

[0065] GPNMB: glycoprotein non-metastatic melanoma protein B; eGFR: estimated glomerular filtration rate; LDL-c: low density lipoprotein cholesterol; OR: Odds ratio; CI: confidence interval.

[0066] **Discussion**

[0067] In this Example, we analyzed global cardiac transcriptomic data from the Heart Failure-HMDP study as a strategy to identify novel plasma biomarkers for heart failure. We found that cardiac transcripts of established HF plasma biomarkers, including TIMP1 and galectin-3, were differentially expressed in ISO-treated mouse hearts and correlated with left ventricular dilation compared to the control group. We identified *Gpnmb* as an attractive candidate based on similar properties and confirmed its upregulation in the MAGNet human heart failure transcriptome collection. Next, we confirm the upregulation of GPNMB protein levels in ISO and TAC mice. Thereafter, we examined plasma GPNMB levels in mice treated with ISO and TAC. We found significantly lower levels of circulating GPNMB in the ISO model and a trend towards decrease in the TAC model. This was a surprising finding that could not be explained by a known mechanism. We also investigated whether lower levels of circulating GPNMB were found in human HF patients. Similar to our observation in mice,

circulating plasma GPNMB levels were also lower in patients with HF from the METSIM study compared to the control group. GPNMB is a single pass transmembrane protein, expressed by inflammatory cells and is thought to undergo cleavage such that cleaved extracellular fragment circulates and regarded to be the active fragment. Our observations demonstrating increased GPNMB levels in the heart associated with decreased circulating GPNMB levels suggest that abnormalities of GPNMB processing including cleavage or binding likely explain decreased circulatory levels in HF.

[0068] GPNMB has been shown to play a role in promoting tissue regeneration after muscle, kidney, liver and cerebral ischemia reperfusion injury by regulation of immune/inflammatory responses and suppressing fibrosis (Abe, Uto et al. 2007, Furochi, Tamura et al. 2007, Nakano, Suzuki et al. 2014, Nagahara, Shimazawa et al. 2015). Previous studies using different cardiac injury models have shown that cardiac tissue levels of GPNMB generally increased in response to stress. These include the desmin knockout mouse model (Psarras, Mavroidis et al. 2012), the Theiler's murine encephalomyelitis virus-induced acute viral myocarditis model (Omura, Kawai et al. 2014), and the myocardial infarction rat and mouse models (Jarve, Muhlstedt et al. 2017). In the myocardial infarction model, GPNMB mRNA transcript was up-regulated 17-fold in the peri-infarct (PI) area in the rat and 300-fold in the mouse at 24 hours and 7 days after myocardial infarction, respectively. Approximately 50% of the CD68+ macrophages expressed GPNMB (Jarve, Muhlstedt et al. 2017). Similar to these publications, we observed an upregulation of *Gpnmb* by isoproterenol on average across the HMDP mouse strains, in the MAGNet human heart failure transcriptome data and two different cardiac injury models isoproterenol and transverse aortic constriction in C57BL/6J mice.

[0069] The exact mechanism by which GPNMB exerts its effect on the heart is not clear. Increased GPNMB expression is seen following injury in multiple organs including the heart (Jarve, Muhlstedt et al. 2017) and kidney (Zhou, Zhuo et al. 2017) and GPNMB could be playing organ specific roles in wound healing. In this regard, a study comparing GPNMB-deficient DBA/2J mice and their coisogenic DBA/2J-GPNMB+ relatives, observed that GPNMB appeared to confer increased risk of adverse ventricular modeling with left ventricular dilation and a decrease in fractional shortening after myocardial infarction (Jarve, Muhlstedt et al. 2017). Because GPNMB has been implicated in endothelial adhesion and transendothelial migration (Shikano, Bonkobara et al. 2001), Jarve et al. postulated that GPNMB-deficiency may impair trans-endothelial migration of monocytes from blood to cardiac tissue. Indeed, elevated numbers of monocytes with the proinflammatory Ly6C^{high} phenotype were identified in the blood and bone marrow of GPNMB-deficient mice (Jarve, Muhlstedt et al. 2017). In contrast, the same adverse impact of GPNMB on cardiac

remodeling was not observed after isoproterenol (Jarve, Muhlstedt et al. 2017). This could be related to the fact that isoproterenol infusion is associated with decreased inflammatory infiltrate compared to an acute injury such as myocardial infarction that is associated with an intense inflammatory infiltrate in the heart. Moreover, previous studies have suggested that GPNMB serves as an inflammatory stop signal in HF that inhibits the activation of T lymphocytes by binding syndecan 4 (Chung, Sato et al. 2007), a proteoglycan that is up-regulated in chronic HF (Takahashi, Negishi et al. 2011) and has been previously shown to adversely influence cardiac remodeling (Kojima, Takagi et al. 2001). If true, increased consumption of GPNMB or lower circulating levels of GPNMB could be indicative of more severe HF. Taken together, whether GPNMB expression is deleterious to cardiac remodeling may depend upon the mode of injury, type of inflammatory response present, and local cellular expression versus circulatory levels of GPNMB ectodomain. Additional studies using different cardiac injury models, examining inflammatory response and sites of GPNMB action are needed to fully delineate GPNMB's relationship with cardiac injury and remodeling.

[0070] Until our study, there has been no publication demonstrating the association between plasma levels of GPNMB and heart failure. The observation of directionally opposite changes in biomarker abundance in tissue versus plasma is especially intriguing. GPNMB, also known as osteoactivin, is a highly-glycosylated type I trans-membrane protein of 572 amino acids that has an integrin and a heparin binding motif, an endosomal sorting signal in the cytoplasmic domain, and a polycystic kidney disease domain of unknown function. It is localized at the cell surface and phagosomal membranes, and there is also a secreted variant of the protein that results from ectodomain shedding by the metalloprotease ADAM10 (Furochi, Tamura et al. 2007, Hoashi, Sato et al. 2010, Rose, Annis et al. 2010). Of note, PKC and Ca (2+) intracellular signaling pathways regulate ectodomain shedding from the largely Golgi-modified form of GPNMB in melanocytes (Hoashi, Sato et al. 2010). Ectodomain fragments of GPNMB act as a growth factor to induce matrix metalloprotease-3 (MMP-3) expression via the ERK pathway in fibroblasts in C2C12 myoblast culture (Furochi, Tamura et al. 2007). ADAM10 has been identified as a sheddase capable of releasing the GPNMB/OA ectodomain from the surface of breast cancer cells, which induced endothelial cell migration (Rose, Annis et al. 2010). Taken together, GPNMB ectodomain shedding that contributes to circulatory GPNMB measured in the plasma may be a highly-regulated process.

[0071] Transcriptome data in the HMDP showed that transcript levels of GPNMB were positively correlated with ADAM9 ($r = 0.22$, p-value = 0.029), which is a sheddase of ADAM10, suggesting their co-regulation, while correlation between GPNMB and ADAM10

was not statistically significant. We postulate that recruitment of GPNMB-expressing monocytes to the heart occurs along with elevated levels of ADAM9, leading to increased ADAM10 shedding and decreased number of active ADAM10 molecules at the cell surface, thereby decreasing GPNMB cleavage by ADAM10 and lowering circulating levels of GPNMB. Alternatively, the endosomal regulation of GPNMB cell surface and phagosomal membrane by PKC and Ca (2+) intracellular signaling pathways may determine cell surface expression, ectodomain shedding and circulating levels of GPNMB. We acknowledge that we have not fully addressed the underlying biology for the directionally opposite changes in biomarker abundance in tissue versus plasma.

[0072] Due to random selection rather than matched selection, our human controls were not properly matched to the heart failure cohort by demographics and comorbidities. Therefore, we cannot conclude based on our human data alone that GPNMB level is an independent heart failure risk factor. However, our experiments in mice, using matched littermate controls, however, bolsters our claim that GPNMB may be a useful independent heart failure biomarker. Finally, we found that GPNMB levels in plasma were independent of proBNP levels, suggesting that GPNMB may be predictive of outcomes based on properties that are dissimilar to the most commonly used biomarker for HF. This characteristic of GPNMB may add a prognostic value to existing clinical practice and, therefore, warranting confirmatory investigation in a larger cohort. Additional biomarkers that ascertain various properties of HF may be important addition to the full evaluation of HF susceptibility.

[0073] This Example describes a proof of concept study illustrating the application of systems genetics data for the identification of biomarkers for HF. We have identified GPNMB as a potential plasma biomarker for heart failure based on data in mouse models and one human cohort.

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[0119] Throughout this application various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to describe more fully the state of the art to which this invention pertains.

[0120] Those skilled in the art will appreciate that the conceptions and specific embodiments disclosed in the foregoing description may be readily utilized as a basis for modifying or designing other embodiments for carrying out the same purposes of the present invention. Those skilled in the art will also appreciate that such equivalent embodiments do not depart from the spirit and scope of the invention as set forth in the appended claims.

What is claimed is:

1. A method of detecting heart failure in a subject, the method comprising:

(a) obtaining a biological sample from the subject;

(b) measuring a decreased amount of glycoprotein non-metastatic melanoma protein B (GPNMB) in the sample relative to a reference amount of GPNMB; and, optionally,

(c) treating the subject for heart failure when the measured amount of GPNMB is decreased.

2. A method of treating heart failure in a subject, the method comprising:

(a) obtaining a biological sample from the subject;

(b) measuring a decreased amount of GPNMB in the sample relative to a reference amount of GPNMB; and

(c) treating the subject for heart failure when the measured amount of GPNMB is decreased relative to the reference amount of GPNMB.

3. A method of screening for heart failure in a subject, the method comprising:

(a) obtaining a biological sample from the subject;

(b) measuring the amount of GPNMB in the sample relative to a reference amount of GPNMB; and

(c) classifying the subject as having heart failure if the measured amount of GPNMB is decreased relative to the reference amount of GPNMB.

4. The method of claim 1, 2, or 3, wherein the sample comprises plasma, serum, or blood.

5. The method of any of the preceding claims, wherein the treating comprises administering a heart failure medication, implanting a device, and/or performing a surgical intervention.

6. The method of claim 5, wherein the heart failure medication comprises an angiotensin-converting enzyme (ACE) inhibitor, an angiotensin-II antagonist, a beta-blocker, an If channel blocker, an aldosterone antagonist, a hydralazine and isosorbide dinitrate, and/or a diuretic.

7. The method of any of the preceding claims, wherein the reference amount of GPNMB is obtained from a healthy, normal control subject.

8. The method of any of the preceding claims, wherein the measuring comprises an immunoassay.

9. The method of claim 8, wherein the immunoassay is an enzyme-linked immunosorbent assay (ELISA).

10. The method of claim 1, 2, or 3, wherein the amount of GPNMB in the sample relative to a reference amount of GPNMB is decreased by 20%.

11. The method of claim 1, 2, or 3, wherein the amount of GPNMB in the sample relative to a reference amount of GPNMB is decreased by 30%.

12. A method of detecting heart failure in a subject, the method comprising:

(a) obtaining an expression product of a gene selected from Serpina3n, AI593442, Gpnmb, Snai3, Spp1, Lox, Gnb3, Catnal1, Retnla, Cdo1, BC020188, BC025833, Angptl7, Pacrg, Arhgdig, Ms4a7, Ccl8, 2310007A19Rik, and Lgals4 in a sample obtained from the subject;

(b) measuring the amount of expression product of the gene in the sample relative to a reference amount; and

(c) classifying the subject as having heart failure if the measured amount of expression product in the sample is decreased relative to the reference amount.

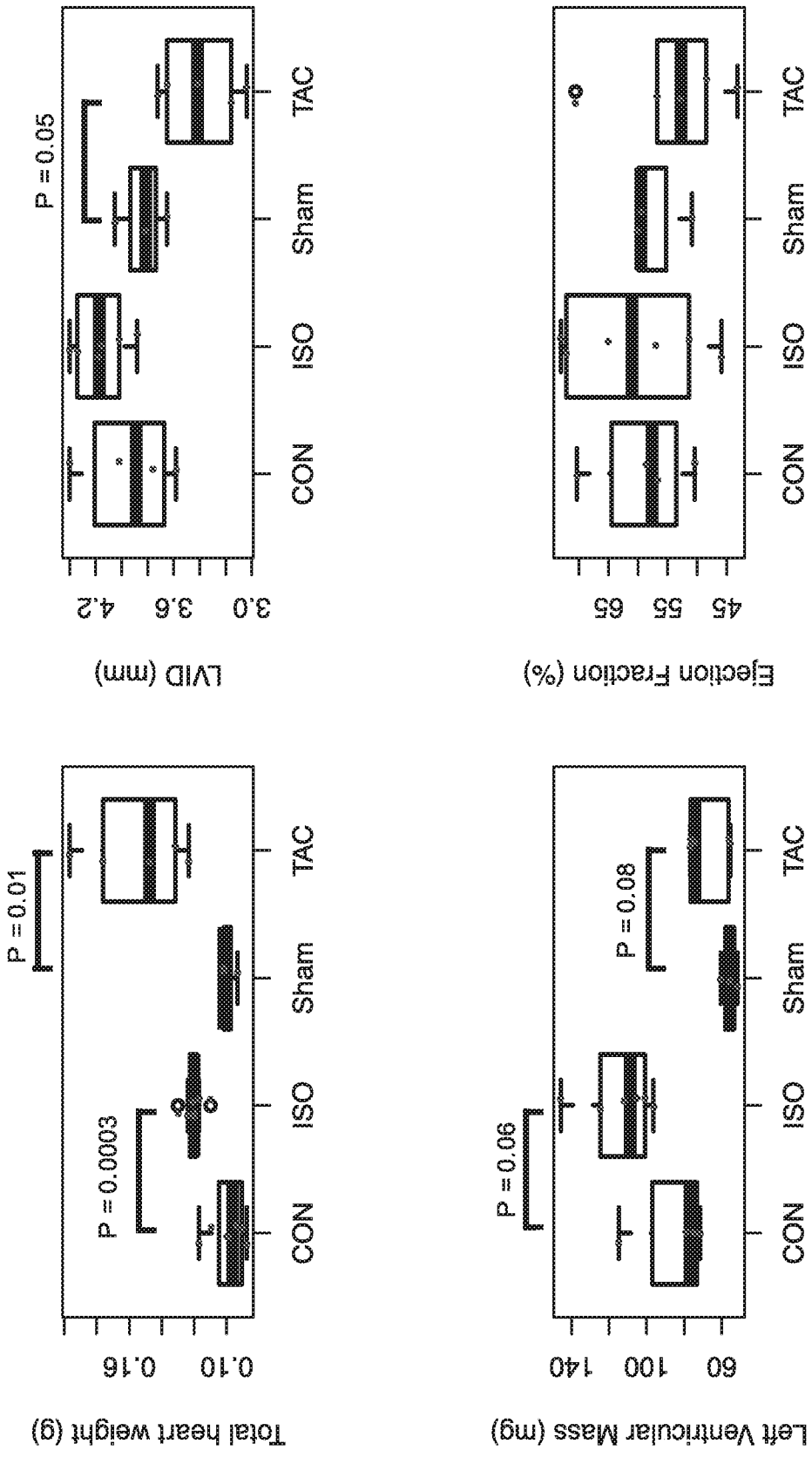
13. The method of claim 12, further comprising treating the subject for heart failure.

14. The method of claim 12 or 13, wherein the reference amount of expression product is obtained from a healthy, normal control subject.

15. The method of claim 12, 13, or 14, wherein the expression product is a protein.

16. The method of claim 15, wherein the measuring comprises an immunoassay.

17. The method of claim 16, wherein the immunoassay is an enzyme-linked immunosorbent assay (ELISA).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3.6
4.2
P = 0.05
CON
ISO
Sham
TAC
Ejection Fraction (%)
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55
65
CON
ISO
Sham
TAC
Total heart weight (g)
0.10
0.16
P = 0.01
P = 0.0003
CON
ISO
Sham
TAC
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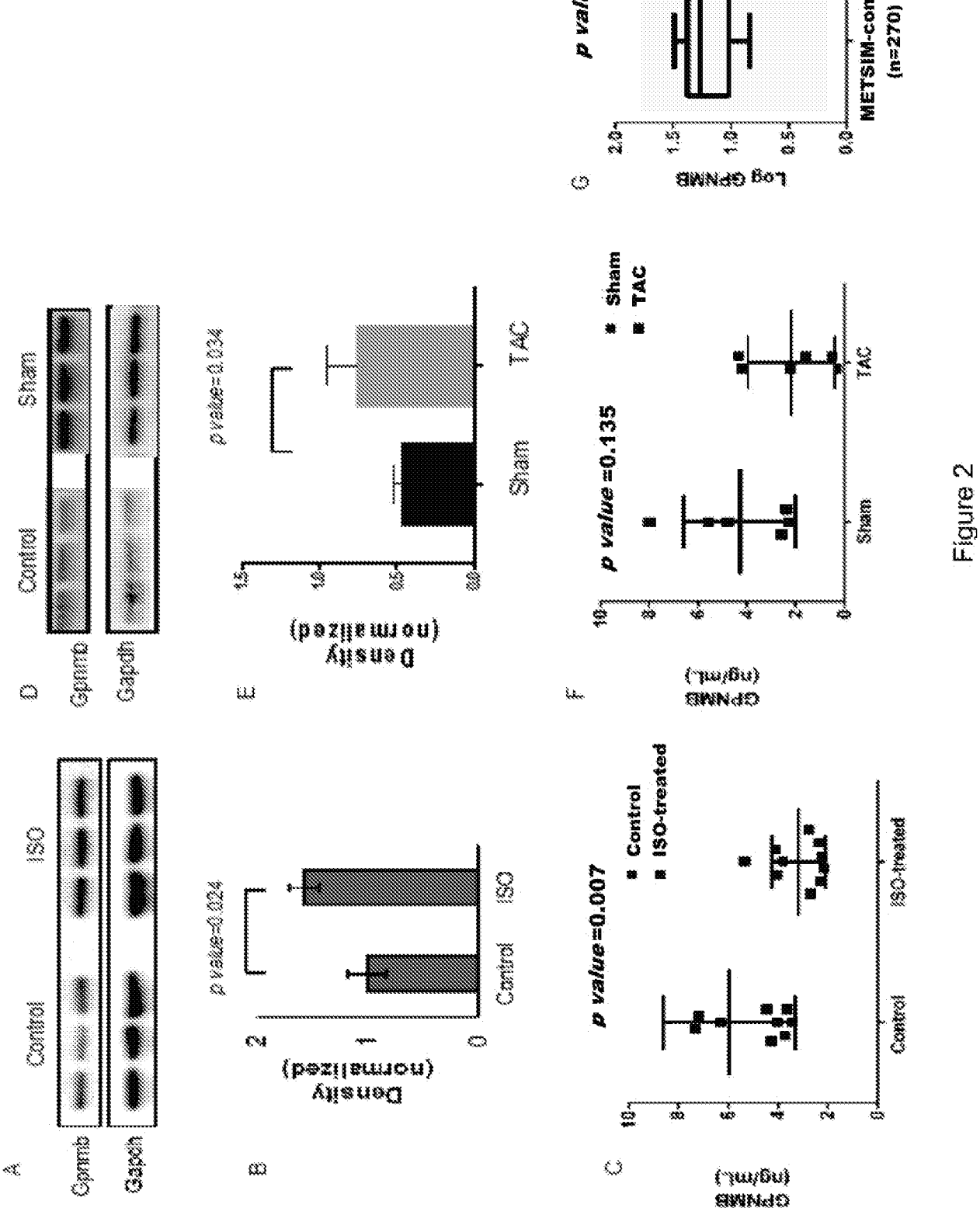
100
140
P = 0.06
P = 0.08
CON
ISO
Sham
TAC

Figure 1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Gpnmb
Gapdh
B
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Density (normalized)
Control
ISO
C
p value=0.007
Control
ISO-treated
GPNMB (ng/mL)
D
Control
Sham
Gpnmb
Gapdh
E
p value=0.034
Density (normalized)
Sham
TAC
F
p value =0.135
Sham
TAC
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G
p value <0.0001
Log GPNMB
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METSIM-HF (n=119)

Figure 2

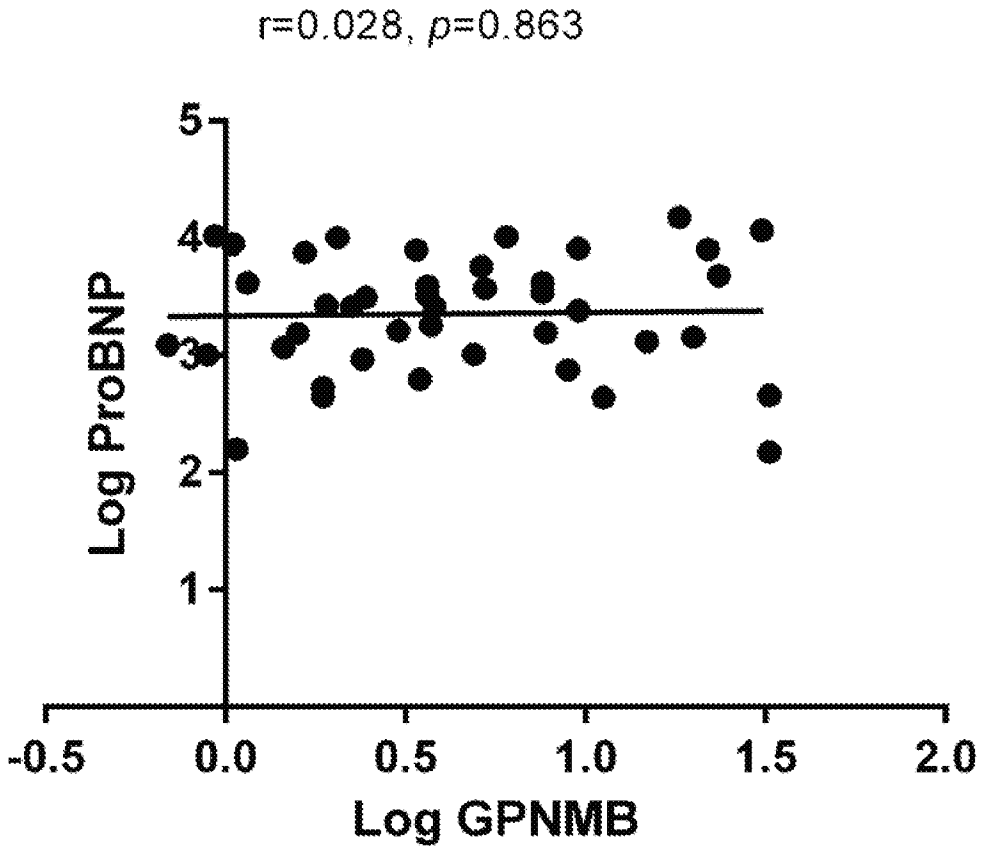

Figure 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 19/49234

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

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

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

3. ☒ Claims Nos.: 5-9, 15-17
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

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

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

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 19/49234

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/6876, C12Q 1/6883 (2019.01)
CPC - C12Q 2600/158, C12Q 2600/106, A61K 38/1841, A61K 39/39558, G01N 2800/52

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)
See Search History Document

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	GUPTA "Translating Mouse Systems Genetics to Discovery in Human Disease" 2017 [online] [Retrieved on 9 October 2019] Retrieved from website < URL: https://escholarship.org/uc/item/9g93k63j > especially, page 87, para 3; page 90, para 1-2; page 92 para 2-3; Fig. 3	1, 3, 4/(1, 3), (10-11)/(1, 3), 12, 14/12 --- 2, 4/2, (10-11)/2, 13, 14/13
Y	US 2014/0031411 A1 (BADER et al.) 30 January 2014 (30.01.2014) Abstract; claim 1; claim 11	2, 4/2, (10-11)/2, 13, 14/13

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 October 2019	27 NOV 2019
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

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