Proteomic Analysis of Coronary Sinus Serum Reveals Leucine-Rich α2-Glycoprotein as a Novel Biomarker of Ventricular Dysfunction and Heart Failure

Chris J. Watson, PhD; Mark T. Ledwidge, PhD; Dermot Phelan, MB; Patrick Collier, MB; Jennifer C. Byrne, PhD; Michael J. Dunn, PhD; Kenneth M. McDonald, MD*; John A. Baugh, PhD*

Background—Heart failure (HF) prevention strategies require biomarkers that identify disease manifestation. Increases in B-type natriuretic peptide (BNP) correlate with increased risk of cardiovascular events and HF development. We hypothesize that coronary sinus serum from a high BNP hypertensive population reflects an active pathological process and can be used for biomarker exploration. Our aim was to discover differentially expressed disease-associated proteins that identify patients with ventricular dysfunction and HF.

Methods and Results—Coronary sinus serum from 11 asymptomatic, hypertensive patients underwent quantitative differential protein expression analysis by 2-dimensional difference gel electrophoresis. Proteins were identified using mass spectrometry and then studied by enzyme-linked immunosorbent assay in sera from 40 asymptomatic, hypertensive patients and 105 patients across the spectrum of ventricular dysfunction (32 asymptomatic left ventricular diastolic dysfunction, 26 diastolic HF, and 47 systolic HF patients). Leucine-rich α2-glycoprotein (LRG) was consistently overexpressed in high BNP serum. LRG levels correlate significantly with BNP in hypertensive, asymptomatic left ventricular diastolic dysfunction, diastolic HF, and systolic HF patient groups (P≤0.05). LRG levels were able to identify HF independent of BNP. LRG correlates with coronary sinus serum levels of tumor necrosis factor-α (P=0.009) and interleukin-6 (P=0.021). LRG is expressed in myocardial tissue and correlates with transforming growth factor-βR1 (P<0.001) and α-smooth muscle actin (P=0.025) expression.

Conclusions—LRG was identified as a serum biomarker that accurately identifies patients with HF. Multivariable modeling confirmed that LRG is a stronger identifier of HF than BNP and this is independent of age, sex, creatinine, ischemia, β-blocker therapy, and BNP. (Circ Heart Fail. 2011;4:188-197.)

Key Words: heart failure ▪ hypertension ▪ natriuretic peptides ▪ leucine-rich α2-glycoprotein

Although therapies for heart failure (HF) have improved, it is clear that effective prevention strategies will have the most important impact on the concerning epidemiology of this syndrome. In this regard, it is essential to complement effective risk factor control with the earliest possible identification of those at risk for progressive ventricular dysfunction and HF. Hypertensive heart disease (HHD) is a well-described example of progressive ventricular dysfunction. Currently, it is not known why a certain proportion of patients with hypertension develop diastolic dysfunction (DD) and others do not. Furthermore, the early diagnosis of the transition to HF is critical and can be clinically challenging. A better understanding of the pathophysiological signals at play may allow for more precise diagnostic tests to define the onset of HF. This natural history is not simply explained by the degree of blood pressure control and probably involves complex disease mechanisms still not fully understood. The ability to identify which asymptomatic hypertensive patients will acquire a more detrimental disease phenotype would have a significant impact on treatment strategies and disease monitoring for this prevalent and important disease.

Clinical Perspective on p 197

B-type natriuretic peptide(s) (BNP) have been shown by us and others to effectively identify various stages of DD in HHD and also to be of prognostic importance in established HF. On the basis of the current literature on BNP, we hypothesize that elevated levels of this peptide in a hypertensive population identifies a more concerning natural history and that analysis of coronary sinus (CS) serum in this...
population may provide further insight into early disease recognition, disease stimuli, and potential therapeutic interventions. We sought to develop this hypothesis by examining the CS serum proteome of a subset of asymptomatic hypertensive patients that were stratified according to BNP levels. The method chosen was 2-dimensional difference gel electrophoresis (2D-DIGE) because of its quantification strengths and proven success in small discovery cohorts.8,9 Two dimensional DIGE is also capable of indicating posttranslational modifications based on isoelectric point shifting. This screening tool that facilitates the dissection of the serum proteome10 identified leucine-rich α2-glycoprotein (LRG) as being over-expressed in asymptomatic patients with elevated BNP, who are at risk of development of HF.

LRG was identified in 1977 as a trace component of human serum,11 and resolution of the primary structure in 1985 indicated that it existed as a single polypeptide chain of approximately 45 kDa.12 The precise function of LRG has yet to be defined, but evidence to date suggests that it is associated with inflammatory responses and neutrophil differentiation13–17 as well as cellular responses to the profibrotic cytokine transforming growth factor (TGF)-β.18,19

A growing body of data suggests that myocardial fibrosis is a central abnormality in the pathogenesis of HHD, DD, and the development of HF.20–22 The factors driving myocardial fibrosis are yet to be fully elucidated but probably involve an exaggerated inflammatory response. The potential biological role of LRG in these processes prompted us to investigate the diagnostic utility of LRG across the spectrum of ventricular dysfunction and HF.

Methods

Patient Recruitment and Sample Collection

Patients

All subjects gave written informed consent to participate in the study. The Ethics Committee at St Vincent’s University Hospital approved the study protocols, which conformed to the principles of the Helsinki Declaration. The study populations reported consist of 145 white patients divided into 4 subgroups: asymptomatic hypertension (n=40) (Table 1), asymptomatic left ventricular diastolic dysfunction (LVDD) (n=32), diastolic HF (DDH) (n=26), and systolic HF (SHF) (n=47) (Table 2); cardiac biopsies were taken from 40 patients undergoing heart surgery. The initial discovery phase of the study used the asymptomatic hypertensive patients. The remaining patient cohorts, LVDD, DHF, and SHF, were used in the validation arm of the study. These various cohorts represent the different phases of the natural history of hypertensive heart disease.

Asymptomatic, hypertensive patients had normal systolic function with left ventricular ejection fraction ≥50%. Patients in the LVDD group had Doppler echocardiographic evidence of DD but exhibited no clinical symptoms of HF. The diagnosis of DHF was based on the presence of all of the following criteria: 1 hospitalization for proven New York Heart Association (NYHA) class IV HF (confirmed by a consultant cardiologist) and continued symptoms of at least NYHA class II and left ventricular ejection fraction ≥45% with Doppler echocardiographic abnormalities of DD without significant evidence of valvular disease. The diagnosis of SHF was based on symptoms and clinical signs of HF and left ventricular ejection fraction ≤45%.

Exclusion criteria included patients with unstable symptoms of HF, those with renal insufficiency (serum creatinine ≥200 µmol/L), and those with clinical evidence of infection. Patients who underwent recent surgery or had recent physical trauma (<6 months) were also excluded. All patients underwent appropriate clinical and laboratory evaluation to identify exclusion criteria and suitability for this study.

Doppler Echocardiography

Doppler echocardiography assessment was performed by a single-blinded operator according to the guidelines laid down by the American Society of Echocardiography. Restrictive-like filling patterns are severe forms of diastolic dysfunction and are defined from Doppler echocardiographic indices as deceleration time <160 ms with ≥1 of the following: left atrial size >5 cm, E/A ratio >1.5, and intraventricular relaxation time <70 ms.

CS and Peripheral Blood Sampling

CS sampling was performed in the asymptomatic hypertensive population during routine clinically indicated cardiac catheterization for suspected ischemic chest pain. These patients did not have unstable angina. Peripheral venous blood samples were obtained during clinical assessment. Serum samples were obtained after centrifugation at 2500 g for 10 minutes at 4°C. Samples were aliquoted and stored at −80°C until required. All steps from collection to storage were carried out at 4°C when possible and within a time frame of <30 minutes. Each serum sample underwent no more than 3 freeze/thaw cycles before analysis.

Table 1. Baseline Demographics of Asymptomatic, Hypertensive Patients With Normal and Elevated (>100 pg/mL) BNP

<table>
<thead>
<tr>
<th>Variable</th>
<th>Normal BNP (n=31)</th>
<th>Elevated* BNP (n=9)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>64±10</td>
<td>70±7</td>
<td>P=0.094</td>
</tr>
<tr>
<td>Sex, male, n (%)</td>
<td>17 (55)</td>
<td>1 (11)</td>
<td>P=0.023</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>28±6</td>
<td>22±14</td>
<td>P=0.715</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>149±20</td>
<td>151±14</td>
<td>P=0.629</td>
</tr>
<tr>
<td>DBP, mm Hg</td>
<td>77±11</td>
<td>77±11</td>
<td>P=0.939</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>69±12</td>
<td>69±12</td>
<td>P=0.648</td>
</tr>
<tr>
<td>Ejection fraction</td>
<td>66±9</td>
<td>61±10</td>
<td>P=0.208</td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
<td>9 (29)</td>
<td>1 (11)</td>
<td>P=0.404</td>
</tr>
<tr>
<td>Biochemical parameters</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium, mmol/L</td>
<td>138±4</td>
<td>139±2</td>
<td>P=0.543</td>
</tr>
<tr>
<td>Potassium, mmol/L</td>
<td>4.1±0.4</td>
<td>4.3±0.5</td>
<td>P=0.224</td>
</tr>
<tr>
<td>Urea, mmol/L</td>
<td>6.2±1.7</td>
<td>6.1±1.8</td>
<td>P=0.899</td>
</tr>
<tr>
<td>Creatinine, µmol/L</td>
<td>93±23</td>
<td>81±22</td>
<td>P=0.252</td>
</tr>
<tr>
<td>Hemoglobin, g/dL</td>
<td>13.4±1.8</td>
<td>12.1±1.5</td>
<td>P=0.057</td>
</tr>
<tr>
<td>WCC, 10^9/L</td>
<td>7.0±2.1</td>
<td>7.8±3.1</td>
<td>P=0.404</td>
</tr>
<tr>
<td>Platelets, 10^9/L</td>
<td>251±66</td>
<td>263±42</td>
<td>P=0.581</td>
</tr>
<tr>
<td>Peripheral BNP, pg/mL</td>
<td>24 (11:37)</td>
<td>244 (137:487)</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>CS BNP, pg/mL</td>
<td>43 (23:79)</td>
<td>588 (420:720)</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Medications</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACEI or ARB, n (%)</td>
<td>22 (71)</td>
<td>3 (33)</td>
<td>P=0.057</td>
</tr>
<tr>
<td>β-blocker, n (%)</td>
<td>14 (45)</td>
<td>8 (89)</td>
<td>P=0.023</td>
</tr>
<tr>
<td>Diuretic, n (%)</td>
<td>7 (23)</td>
<td>4 (44)</td>
<td>P=0.227</td>
</tr>
<tr>
<td>Statin, n (%)</td>
<td>21 (68)</td>
<td>4 (44)</td>
<td>P=0.234</td>
</tr>
<tr>
<td>Antplatelet agents, n (%)</td>
<td>23 (74)</td>
<td>7 (78)</td>
<td>P=1.000</td>
</tr>
</tbody>
</table>

Data are presented as mean±SD or median (interquartile range) unless otherwise specified.

BMI indicates body mass index; HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; WCC, white cell count; LDL, low-density lipoprotein; HDL, high-density lipoprotein; Tg, triglycerides; and ACE, angiotensin-converting enzyme.

*Elevated BNP >100 pg/mL.
Table 2. Baseline Demographics of Patients With Asymptomatic Left Ventricular Dysfunction, DHF, and SHF

<table>
<thead>
<tr>
<th>Variable</th>
<th>Asymptomatic Left Ventricular Dysfunction</th>
<th>DHF</th>
<th>SHF</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>32</td>
<td>26</td>
<td>47</td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td>68±9</td>
<td>72±11</td>
<td>70±9</td>
<td>0.285</td>
</tr>
<tr>
<td>Sex, male</td>
<td>24 (75)</td>
<td>14  (54)</td>
<td>31 (66)</td>
<td>0.240</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>149±20</td>
<td>138±20</td>
<td>133±22</td>
<td>0.050</td>
</tr>
<tr>
<td>DBP, mm Hg</td>
<td>77±11</td>
<td>73±13</td>
<td>72±12</td>
<td>0.182</td>
</tr>
<tr>
<td>Ischemic etiology</td>
<td>11 (34)</td>
<td>12 (46)</td>
<td>33 (70)</td>
<td>0.010</td>
</tr>
<tr>
<td>Smoking</td>
<td>6 (19)</td>
<td>1 (4)</td>
<td>25 (53)</td>
<td>0.134*</td>
</tr>
<tr>
<td>Raised cholesterol</td>
<td>21 (66)</td>
<td>15 (58)</td>
<td>20 (43)</td>
<td>0.114</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>4 (13)</td>
<td>4 (15)</td>
<td>10 (21)</td>
<td>0.575</td>
</tr>
<tr>
<td>Ejection fraction, %</td>
<td>66.9±9.5</td>
<td>63.3±13.7</td>
<td>33.9±5.9</td>
<td>0.001</td>
</tr>
<tr>
<td>Left atrial volume, mL</td>
<td>54.8±23.7</td>
<td>86.5±31.2</td>
<td>90.4±34.7</td>
<td>0.001</td>
</tr>
<tr>
<td>Left ventricular mass, g</td>
<td>203.7±52.7</td>
<td>226.1±75.3</td>
<td>244.3±61.1</td>
<td>0.050</td>
</tr>
</tbody>
</table>

Biochemical indicators

| LRG, ng/mL                | 35.4±5.4                                 | 38.5±5.3 | 45.2±9.6 | 0.001   |
| Urea, mmol/L              | 5.9±1.6                                  | 8.9±4.7 | 10.6±45.1 | 0.010   |
| Creatinine, µmol/L        | 100.6±26.0                               | 115.1±48.2 | 137±55 | 0.010   |
| Sodium, mmol/L            | 138.6±4.2                                | 139.5±4.6 | 138.5±4.4 | 0.601   |
| Potassium, mmol/L         | 4.0±0.5                                  | 4.1±0.5 | 4.3±0.6 | 0.051   |
| BNP, pg/mL                | 109.3±137.8                              | 264.7±181.7 | 345.5±426.2 | 0.050   |

Medications

| ACEI or/and ARB           | 24 (75)                                  | 20 (77) | 39 (83) | 0.662   |
| β-blocker                 | 17 (53)                                  | 17 (66) | 37 (79) | 0.050   |
| Aldosterone antagonist    | 2 (6)                                    | 0       | 6 (13) | 0.961*  |
| Statin                    | 19 (59)                                  | 13 (50) | 26 (62) | 0.775   |
| Loop diuretic             | 0                                        | 22 (85) | 42 (89) | 0.001*  |

Data are presented as mean±SD or median (interquartile range) unless otherwise specified. SBP indicates systolic blood pressure; DBP, diastolic blood pressure; ACE, angiotensin-converting enzyme; ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker; and NS, not significant.

*To overcome too few arguments, DHF and SHF were combined for analysis.

Two-Dimensional DIGE

A full description of the proteomic work flow is given in the online-only Data Supplement file. Briefly, CS serum from 11 asymptomatic, hypertensive patients (BNP <100 pg/mL, n=5; BNP ≥100 pg/mL, n=6) underwent affinity depletion of the 6 most abundant proteins (albumin, immunoglobulin G, immunoglobulin A, transferrin, haptoglobin, and α1-antitrypsin) using a MARS-Hu6 high-performance liquid chromatography immunoaffinity column (Agilent Technologies). Quantitative differential protein expression analysis was carried out using 2D-DIGE, based on the labeling of sample proteins using fluorescent CyDye reagents. Each depleted serum sample was labeled with Cy5. A pooled serum standard was generated and labeled with Cy3 and was included in each 2D-DIGE run as a reference. Differentially expressed proteins were identified after image scanning and quantitative computer image analysis (Progenesis SameSpots, Nonlinear Dynamics). Selected protein spots that were significantly different between the 2 groups were isolated from silver stained gels then trypsin digested before identification by mass spectrometry.

Serum Biomarker Assessment

BNP was quantified using a Triage meter BNP assay (Biosite Inc). Serum levels of LRG were quantified using a Human LRG Assay Kit (IBL) according to the manufacturer’s instructions. The inflammatory cytokines interleukin (IL)-6 and tumor necrosis factor (TNF)-α were quantified in the CS serum using an ultrasensitive immunoassay with electrochemiluminescence detection (Meso Scale Discovery), as instructed by the manufacturer. Additional information on these assays and their performances are detailed in the online-only Data Supplement.

Quantitative Real-Time Polymerase Chain Reaction Analysis

Total RNA was isolated from human primary coronary artery endothelial (HCAEC) cells (Promocell) and human primary ventricular cardiac fibroblasts (HVCF) (ScienCell Research Laboratories), using a NucleoSpin RNA II Kit (Macherey-Nagel) according to the manufacturer’s instructions. HCAEC cells were confirmed by the supplier to be von Willebrand factor positive, CD31 positive, Dil-Ac-LDL uptake positive, and confirmed by the supplier to be von Willebrand factor positive, CD31 positive, Dil-Ac-LDL uptake positive, and α-smooth muscle actin negative; HVCF cells were shown to be fibronectin positive. Right atrial tissue was collected from patients with ischemic etiology confirmed by the supplier to be von Willebrand factor positive, CD31 positive, Dil-Ac-LDL uptake positive, and confirmed by the supplier to be von Willebrand factor positive, CD31 positive, Dil-Ac-LDL uptake positive, and α-smooth muscle actin negative; HVCF cells were shown to be fibronectin positive. Right atrial tissue was collected from patients with ischemic etiology undergoing coronary artery bypass surgery. Immediately after biopsy collection, the tissue was stored in Allprotect Tissue Reagent (Qiagen) and RNA was subsequently isolated using AllPrep DNA/RNA Mini-Kit (Qiagen) according to the manufacturer’s instructions. RNA (600 ng) was reverse-transcribed using SuperScript II RT (Invitrogen) before quantitative real-time polymerase chain reaction performed as previously described.21 Gene-specific primers used are as follows: LRG, 5'-GTCTCTTTGGAACGACACGC-3' (forward), 5'-AGGGTGTTGACAGGAATGG-3' (reverse); TGF-βR1, 5'-ATGGCTGAGCAAGAGTCTT-3' (forward), 5'-AAA-CCTGAGCCAGAACCCTGA-3' (reverse); α-smooth muscle actin (forward), 5'-GGTCCCGAACACGAGGG-3' (reverse), 5'-GGGTCTGAGCAGGAGTTTGA-3' (reverse).
(ASMA), 5'-CTTACTACTGCTGAGCGTGA-3' (forward), 5'- ACGTTCTATTCCAGTGGTTG-3' (reverse); and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase, 5'-ACAGTCACGC- CGCATTCCTT-3' (forward), 5'-ACGACAAATCCGTTGACTC-3' (reverse). The samples were quantified using the delta computed tomography method. Samples were electrophoresed on a 2% polyacrylamide gel using SybrSafe (Invitrogen) for visualization.

**Immunohistochemical Analysis of LRG**

For the purpose of immunohistochemical detection and localization of LRG protein within the myocardium, 3 right atrial tissue samples were collected from patients with multivessel ischemic heart disease undergoing coronary artery bypass surgery. Tissue specimens were immediately washed in PBS and fixed in 10% formalin, followed by paraffin embedding. Tissue sections (5 μm) were treated with 0.01 mol/L citrate buffer at pH 6 for 20 minutes at 95°C before incubation with a rabbit anti-LRG polyclonal antibody (Atlas Antibodies) or the corresponding immunoglobulin G control at an equivalent concentration. Immunoreactivity was detected with EnVision (Dako). In addition, tissue sections were stained with hematoxylin and eosin for morphological assessment of the cardiac tissue. Slides were imaged using the Aperio ScanScope digital scanner and visualized using VisionScope V10 (Aperio Technologies).

**Statistical Analysis**

Differences in 2D-DIGE spot volumes between low and high BNP groups were determined using the Student t test, with probability values ≤0.05 considered statistically significant. For continuous variables, summary statistics are presented as the mean±SD or median and 25th to 75th percentiles. Categorical variables are presented as frequencies and percentages (in parenthesis). Comparisons between the normal and elevated BNP groups were made using an independent t test, Wilcoxon rank-sum test, or χ² test where appropriate. The relationship between BNP and LRG was assessed using the Pearson correlation coefficient for variables that were approximately normally distributed and using log-transformation of variables with nonnormal distribution. Spearman rank-order correlation was used for analysis of nonnormal tissue gene expression data. Receiver operating characteristic (ROC) curves were plotted to assess the usefulness of LRG and BNP in identifying restrictive-like filling patterns and DHF in patients without SHF. Furthermore, we examined the ability of LRG and BNP to identify any HF from non-HF patients. Univariable and multivariable analyses were conducted using binary logistic regression to determine the ability of LRG to identify the presence of restrictive-like filling patterns or HF as the outcome variables. For multivariable analysis, the probability value of the partial likelihood ratio test was used to confirm if a covariate was significant and the coefficients of the remaining variables were assessed to determine if important (>20%) changes had occurred on variable exclusion. All statistical calculations were performed using SPSS V.16 software.

**Results**

**Proteomic Analysis of CS Sera in Asymptomatic, Hypertensive Patients With High and Low BNP**

Comparison of the CS serum proteome of 11 asymptomatic hypertensive patients using 2D-DIGE and quantitative computer image analysis of 3D spot volumes revealed 949 different protein spots on the 2D gels, of which 117 were differentially expressed. Two neighboring protein spots, Spot ID-0416 and Spot ID-0442, were differentially expressed in high BNP serum by 1.8- and 2.1-fold, respectively (P <0.05). The protein spots were excised from the 2D gels and identified using mass spectrometry as LRG (isoelectric point, 6.5; molecular weight, 38154.13 Da) (Figure 1A). The 3 adjacent spots that were not differentially expressed (Spots 3, 4, and 5, Figure 1A) but were in the same row as the differentially expressed LRG spots were also identified. These 3 neighboring spots were also found to be LRG. This train of LRG spots (Spots 1 to 5) represents variants of LRG based on the different migratory patterns through the 2D gels. The existence of LRG variants is potentially explained by posttranslational glycosylation.24 Pooling of the 3D spots volumes from the 2 differentially expressed LRG variants revealed a significant 2-fold increase in the high BNP group (online-only Data Supplement Figure 1). The potential biological role of LRG prompted our focus on this protein. We verified our 2D-DIGE observation in the 11 asymptomatic hypertensive patients using an enzyme-linked immunosorbent assay (ELISA) (P <0.05) (Figure 1B). We further examined CS serum levels of LRG in an expanded cohort totaling 40 asymptomatic hypertensive patients (Table 1). We found that LRG levels significantly correlated with CS BNP levels (r =0.32, P <0.05), suggesting that LRG is associated with patients at risk of development of DD and HF (Figure 1C). Serum LRG levels were not associated with the extent of coronary artery disease, as shown by angiography (online-only Data Supplement Figure 2).

**Quantification of LRG Levels Across the Spectrum of Ventricular Dysfunction**

Serum levels of LRG were further quantified in additional patient cohorts across the spectrum of asymptomatic LVDD, DHF, and SHF (Table 2). LRG levels within the peripheral serum significantly correlated with BNP levels (r =0.47, P <0.001) (Figure 2A). A significant incremental increase in serum LRG levels was detected between asymptomatic LVDD and DHF (P <0.05), asymptomatic LVDD and SHF (P <0.001), and DHF and SHF (P <0.01) (Figure 2B). Serum LRG correlated with echocardiography parameters. When asymptomatic hypertensive patients, asymptomatic LVDD patients, DHF patients, and SHF patients were analyzed collectively, it was found that LRG significantly correlated with left ventricular mass index (r =0.32, P <0.001; online-only Data Supplement Figure 2).

**LRG Identifying Restrictive-Like Filling Patterns and HF**

ROC curve analyses showed that LRG and BNP were able to significantly identify patients with restrictive-like filling patterns (n =15 versus n =83 normal or mild/moderate diastolic dysfunction; area under the curve [AUC], 0.82, 0.85, respectively; both P <0.001) and DHF (n =26 DHF versus n =72 no-HF; AUC, 0.87, 0.81, respectively, both P <0.001). Furthermore, both LRG and BNP were useful in identifying any HF in the data set (n =73 HF versus n =72 no-HF; AUC, 0.86, 0.85, respectively; both P <0.001, Figure 2C). Multivariable analysis showed that LRG ability to identify any HF is independent of age, sex, creatinine, and BNP (adjusted hazard ratio, 1.460; 95% confidence interval, 1.183 to 1.801; P <0.0001). In this model, BNP was not able to discriminate the HF population from the no-HF population (adjusted hazard ratio, 1.003; 95% confidence interval, 0.999 to 1.006; P =0.171). Importantly, LRG continued to be able to significantly identify HF when the multivariable analysis was
further adjusted for beta blocker usage and ischemic events (adjusted hazard ratio, 1.755; 95% confidence interval, 1.022 to 3.013; P<0.05).

**Relationship Between LRG and Inflammation**
CS serum levels of LRG significantly correlated with the inflammatory cytokines TNF-α (r=0.44, P=0.009) and IL-6 (r=0.38, P=0.021) in an asymptomatic hypertensive population (Figure 3A).

**Relationship Between Cardiac Tissue LRG and Expression of Fibrogenic Genes**
Polymerase chain reaction analysis of LRG expression was assessed in commercially available human primary cells that originated from cardiac tissue, right atrial biopsy tissue samples, and human primary neutrophils. In vitro evidence of LRG mRNA production was detected in primary HCAEC and VHCF (Figure 3B). Evidence of myocardial tissue being a potential source of LRG was also confirmed (Figure 3B). The relationship between cardiac tissue LRG, TGF-β receptor, and the myofibroblast-related gene ASMA was assessed in 40 right atrial tissue samples using quantitative real-time polymerase chain reaction. Cardiac tissue LRG significantly correlated with both TGF-β receptors (r=0.61, P<0.001) and ASMA (r=0.36, P=0.025) (Figure 3C). Confirmatory evidence of LRG protein being localized within the myocardium is highlighted in Figure 3E. Examination of 3 cardiac biopsies revealed that cardiac myocytes, which are apparent as the largest cell type within the
tissue section, exhibited positive immunostaining for LRG. The distribution and intensity of LRG within the myocytes was varied, with specific cytoplasmic and membranous expression detected. Cardiac fibroblasts, the more numerous but smaller cell type within the heart, exhibiting elongated nuclei, are also evident within Figure 3E, and appear to be negative for LRG protein expression.

Discussion
Natriuretic peptides have been shown by us and others to effectively identify various stages of DD in HHD and also to be of prognostic importance in established HF. Using BNP to stratify a population of asymptomatic hypertensive patients, we sought to identify novel biomarkers that may represent evidence of early myocardial injury, which, in addition, may provide insight into disease pathogenesis, susceptibility to the development of ventricular dysfunction, and potentially guide novel diagnostic and therapeutic strategies. Two-dimensional DIGE analysis of CS serum followed by mass spectrometry revealed exaggerated expression of LRG in patients with elevated BNP (≥100 pg/mL). LRG-specific ELISA validated this finding in CS sera of asymptomatic hypertensive patients. We further examined CS serum levels of LRG in an expanded cohort totaling 40 asymptomatic hypertensive patients and found that LRG levels significantly correlated with CS BNP levels. In this patient cohort, LRG was also found to correlate with expression of the inflammatory cytokines IL-6 and TNF-α. LRG mRNA expression was confirmed in human primary cardiac fibroblasts, primary coronary artery endothelium, and cardiac tissue biopsy samples. Correlation of LRG mRNA with TGF-βR1 and ASMA expression in myocardial tissue suggests a link to fibrogenic pathways. Immunohistochemistry confirmed that LRG protein

Figure 2. A, Correlation between BNP and LRG in patients with LVDD and HF analyzed using ELISA-based methodologies. B, Analysis of LRG in patients with asymptomatic LVDD (n=32, range 25.2 to 43.5), DHF (n=26, range, 27.4 to 47.8), and SHF (n=47, range, 25.5 to 59.5). C, ROC curve comparing LRG and BNP in identifying HF (n=73 HF versus n=72 no HF; AUC, 0.86, 0.85, respectively; both P<0.001).
Figure 3. A, Correlation between CS LRG and CS TNF-α and CS IL-6 in the serum of asymptomatic, hypertensive patients (n=40; TNF-α range, 2.0 to 5.9; IL-6 range, 0.7 to 9.5) using an ELISA approach. B, Evidence of LRG mRNA expression in 4 atrial cardiac tissue samples, primary HCAEC, primary HVCF, and neutrophils that served as a positive control, as indicated by reverse transcriptase–polymerase chain reaction analysis. C, Correlation between LRG and TGF-β1 and α-ASMA cardiac tissue mRNA (n=40). Representative image of right atrial biopsy tissue was used in this study. D, Tissue was stained with hematoxylin and eosin for morphological assessment. E, Immunohistochemical evidence of LRG protein expression within myocardial tissue. Cardiac myocytes (examples indicated with purple arrows) exhibited positive membranous (black arrow) and cytoplasmic immunostaining (gray arrow). Cardiac fibroblast cells (examples indicated with green arrows) appear to be negative for LRG protein expression, as indicated by immunohistochemistry. Images presented are original magnification ×20 and ×40.
was expressed within human myocardial tissue and suggests that cardiac myocytes are the primary tissue source. Further analysis of peripheral serum from an asymptomatic LVDD, DHF, and SHF patient cohort identified LRG as a potential novel biomarker of early and sustained ventricular dysfunction and HF. This observation is supported by ROC curve analysis showing the ability of LRG to significantly identify restrictive filling patterns and HF. In addition, multivariable modeling in this cohort shows that LRG is a stronger indicator of HF than BNP, and this is independent of age, sex, creatinine, ischemia, β-blocker therapy, and BNP. These data suggest that LRG may be a valuable marker of ventricular dysfunction from its earliest stage and may add to our ability to diagnose and monitor patients with HF. Furthermore, with its links to inflammation and fibrogenic pathways, LRG may provide insight into the pathogenesis of this syndrome.

Although there are no data to date to indicate the precise function of LRG, the presence of a leucine at every 7th position in segments of this protein suggest the possibility of forming a leucine-zipper structure, which has been implicated in protein-DNA and protein-protein interaction. Also, LRG has been shown to be associated with neutrophilic differentiation and prevention of lymphocyte apoptosis, whereas in hepatoma cell lines, LRG expression has been shown to be associated with increased susceptibility to TGF-induced growth suppression. Some evidence suggests that LRG may be an acute phase protein. For example, its expression is induced synergistically by IL-6 and TNF in hepatocytes. Mass spectrometry analysis of urine from acute pediatric appendicitis patients has recently identified LRG as a potential biomarker that correlates with the severity of appendicitis. Further, a 9.5-fold enrichment in LRG was shown in diseased appendices, which was accompanied by an 11-fold enrichment in TGF-βR2. Although immunostaining localized LRG to neutrophilic focal lesions in diseased appendices, the association with TGF-βR2 implies a potential overlap with TGF-driven repair mechanisms.

TGF-β-induced fibrogenic responses require dimerization of TGF-βR2 with TGF-βR1 to enable signal transduction and downstream activation of the SMAD2/3 pathway. In support of a role of LRG in cardiac fibrosis, we have provided evidence of LRG protein expression within the myocardium. We have also demonstrated a positive correlation between LRG and TGF-βR1 gene expression. The association between LRG and TGF-induced fibrogenic pathways is further supported by a positive correlation between LRG and ASMA gene expression in myocardial tissue. Given that inflammation potentially drives myocardial injury and precedes the development of fibrosis, it is noteworthy that LRG serum levels were found to correlate with those of IL-6 and TNF-α in asymptomatic, hypertensive patients.

The association between LRG and inflammation as well as echocardiographic parameters of cardiac structure (left ventricular mass index) may reflect a role for LRG in the myocardial response to early injury and the initiation of tissue repair processes. It is the downstream effects of inflammation that probably initiate interstitial disease within the myocardium, which we and others have shown to be a critical pathophysio-
logical process in the development of DD. Indeed, it may be that this proposed inflammatory stimulus may help explain the natriuretic response seen in these patients. As a cardioprotective protein, BNP probably represents a response to early injury.

Our data also demonstrate that LRG may be a valuable biomarker later in the syndrome of ventricular dysfunction when HF is present. We have defined a stepwise increase in LRG levels comparing patients with asymptomatic HHD, those with HF and preserved systolic function, and those with systolic dysfunction HF. The stimulus for LRG in these later phases of ventricular dysfunction is unclear, but it is reasonable to hypothesize that it could again be linked to the low-grade inflammation observed in this syndrome. Natriuretic peptides have some limitations in the diagnosis of HF, such as significant biological variability and dependence on noncardiac influences such as β-blocker use, body mass index, renal function, and age. Therefore, identification of other biomarkers and their use in isolation or with natriuretic peptides may provide a more accurate detection of subclinical disease and indeed prediction of progression, outcome, or response to treatment. Although there is a statistically significant increase in serum LRG levels across patient groups, it should be noted that the magnitude of the increase is subtle. Further work is required to evaluate the performance of LRG as a diagnostic and prognostic biomarker in DD and HF; however, the present data should encourage such work, given the comparable and independent diagnostic power of LRG versus BNP, the current guideline standard for biochemical diagnosis of HF.27

Further work on posttranslational modifications of LRG may also increase the utility of this marker. The presence of LRG in 5 distinct spots on our 2D gels suggests the existence of multiple forms of LRG. This is supported by previous studies that have shown an increase in LRG fucosylation, mannosylation, and sialylation in pancreatic cancer.24 Even though we show a clear association of LRG with HF, it is possible that identification of specific LRG glycosylation patterns may strengthen this observation. The ELISA used in this study potentially recognizes all forms of LRG; therefore, the signal may be diluted by the variants that are not differentially expressed. The development of an LRG variant-specific ELISA may greatly improve the diagnostic power of this marker.

In summary, these novel findings linking LRG with ventricular dysfunction and HF suggest that this protein may have value as a biomarker in this syndrome. In particular, given its expression early in the natural history of this syndrome, LRG may have a role in the identification of those at risk for progression of disease and possibly even provide an insight into the relevant pathophysiological signals at play.

Limitations

As an initial report of the potential role of LRG in ventricular dysfunction and HF, further work is required to clarify many issues not explored in the present study. While defining a differential expression of LRG in those at risk for ventricular dysfunction, the exact role of LRG in disease pathogenesis remains unclear. In addition, we believe that a prospective study in defined patient populations is required to evaluate the biological and analytic variability of LRG. Interestingly, this has previously been highlighted as a potential weakness of natriuretic peptides in certain cohorts.28 Although we have only reported on one of the differentially expressed proteins identified during the 2D-DIGE screen, we are currently isolating additional protein spots to be identified using mass spectrometry. These, too, must be further investigated to determine their disease relevance and ability to perform as potential disease biomarkers. It is possible that a panel of differentially expressed serum proteins may be defined in serum of patients at risk for ventricular dysfunction that may be applied in combination with LRG. It is likely that a multimarker approach will have much greater disease specificity. We provide evidence of cardiac LRG production and expression that suggests the potential association with myocardial disease but does not confirm cardiac specificity. Although LRG is able to identify HF in this study, it must be acknowledged that LRG levels are also increased in other diseases.25,29 This is a limitation that may preclude the use of LRG in the general population as a single marker indicator of HF. Given the difficulties in predicting and diagnosing LVDD and DHF, we suggest that LRG may prove useful in a clinical setting as part of a multimarker approach in combination with routine clinical assessment.

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Disclosures

Dr McDonald received honoraria from Inverness Medical Innovations, Inc. Components of this work have been filed by Drs Watson, Ledwidge, McDonald, and Baugh as part of a patent application for biomarkers in cardiovascular disease.

References

Although therapies for heart failure have improved, it is clear that effective prevention strategies will have the most important impact on the concerning epidemiology of this syndrome. In this regard, it is essential to complement effective risk factor control with the earliest possible identification of those at risk for progressive ventricular dysfunction and heart failure. In this study, leucine-rich α-2-glycoprotein (LRG) is identified as a serum biomarker of early and sustained ventricular dysfunction and heart failure. This observation is supported by ROC curve analysis showing significant predictive power of LRG for restrictive filling patterns and heart failure. In addition, multiparticle modeling shows that LRG is a stronger predictor of heart failure than B-type natriuretic peptide (BNP) and this is independent of age, sex, creatinine, ischemia, β-blocker therapy and BNP. Although further work is required to evaluate the performance of LRG as a diagnostic and prognostic biomarker in diastolic dysfunction and heart failure, the present data should encourage such work given the comparable and independent predictive power of LRG versus BNP, the current guideline standard used in collaboration with the Heart Failure Association of the ESC (HFA) and endorsed by the European Society of Intensive Care Medicine (ESICM). Eur J Heart Fail. 2008;10:933–989.

Proteomic Analysis of Coronary Sinus Serum Reveals Leucine-Rich α2-Glycoprotein as a Novel Biomarker of Ventricular Dysfunction and Heart Failure

Chris J. Watson, Mark T. Ledwidge, Dermot Phelan, Patrick Collier, Jennifer C. Byrne, Michael J. Dunn, Kenneth M. McDonald and John A. Baugh

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SUPPLEMENTAL MATERIAL
Supplemental Methods

Two Dimensional Difference Gel Electrophoresis (2D-DIGE)

Depletion of coronary sinus serum

Eleven coronary sinus (CS) serum samples from asymptomatic hypertensive patients (6 high BNP ≥100pg/ml and 5 low BNP <100pg/ml) were affinity depleted of the six most abundant serum proteins; albumin, immunoglobulin G, immunoglobulin A, transferrin, heptoglobin, and anti-trypsin. These proteins constitute approximately 85-90% of the serum proteome. This was to enhance the sensitivity of downstream analysis and potentially reveal the less abundant pathologically relevant proteins of interest. This was achieved using the human Multiple Affinity Removal System, MARS Hu-6 column (Agilent Technologies), as previously described \(^1\). During depletion, sample carry over was prevented by ensuring that the MARS column was extensively washed and that base line returned to normal prior to depleting the next sample. Samples then underwent ice-cold acetone precipitation due to the large volume of depleted serum in buffer. To ensure maximal acquisition of the precious depleted protein, protein concentrations were not assessed post depletion and prior to acetone precipitation, therefore we are unable to calculate any potential loss between these two stages. However, this step was performed simultaneously with all 11 samples.

Sample labeling with CyDyes

Depleted serum samples were precipitated using ice cold acetone and re-suspended in DIGE Lysis Buffer (9.5 M Urea, 2% CHAPS, 20 mM Tris, pH 8.5). Samples were cleaned up and concentrated using the Ettan 2-D Clean-up Kit (GE Healthcare), and quantified using the Bradford assay using a BSA standard. CS serum samples were adjusted to a concentration of 2µg/µl using DIGE Lysis Buffer and labeled with CyDye DIGE Fluors (minimal dyes) for Ettan DIGE (GE Healthcare). For each sample, 40µg of serum protein was labeled with
320pmol Cy5. In addition, an internal standard was generated by pooling together 40µg of each serum and labeling with Cy3.

**First Dimension isoelectric focusing**

Proteins were separated according to charge (pI) under denaturing conditions using immobilised pH gradients (IPG). For each sample to be analyzed, 40µg of Cy3 labeled internal control was combined with 40µg of Cy5 labeled serum sample and mixed with 2x Sample Buffer (9.5 M Urea, 2% CHAPS, 2% Dithiothreitol (DTT), 1.6% Pharmalyte). Rehydration Buffer (8 M Urea, 0.5% CHAPS, 0.2% DTT, 0.2% Pharmalyte) was added to the sample and overlaid onto a pH 4-7, 24cm IPG Immobiline™ DryStrip (GE Healthcare). Passive in-gel rehydration was allowed to occur overnight in the dark. The IPG strip rehydrated with the sample was focused in the first dimension using an Ettan IPGphor3 Isoelectric Focusing unit (GE Healthcare).

**Second Dimension SDS-PAGE**

Proteins were separated according to their molecular weight under denaturing conditions. IPG strips were equilibrated using two equilibration buffers (6 M Urea, 50 mM TrisCl pH 8.8, 30% (v/v) Glycerol, 2% (w/v) SDS, 1% (w/v) DTT) for 15 minutes followed by (6 M Urea, 50 mM TrisCl pH 8.8, 30% (v/v) Glycerol, 2% (w/v) SDS, 2.5% (w/v) iodoacetamide) for 15 minutes. Equilibrated IPG strips were added to 12% SDS-PAGE gels that were cast using low fluorescent plates. Gels were ran in a PROTEAN Plus Dodeca Cell tank (Bio-Rad) using 1x SDS Electrophoresis Buffer (25 mM Tris, 192 mM Glycine, 0.1% (w/v) SDS) at 1 Watt per gel, while being maintained at 15°C and kept in the dark. Following separation in the second dimension, gel cassettes were scanned using a Typhoon 9410 Variable Mode Imager scanner (GE Healthcare) at a resolution of 100µM for quantitative computer image analysis with Progenesis SameSpots software (Nonlinear
Dynamics). Following software alignment of the 2D-DIGE images, normalization and statistical analysis, differentially expressed protein spots were identified.

**Mass spectrometry and peptide identification**

Preparative 2D-gels loaded with 300µg of depleted CS serum protein were generated for mass spectrometry identification of differentially expressed proteins. 2D-gels were fixed and silver stained using PlusOne Silver Stain Kit (GE Healthcare) according to the manufacturer’s instructions. Protein spots of interest were isolated from the 2D-gels, destained, and trypsin digested. Tryptic peptides were resuspended in 0.1% formic acid. Samples were run on a Thermo Scientific LTQ ORBITRAP XL mass spectrometer connected to an Exigent NANO LC.1DPLUS chromatography system incorporating an autosampler. A high resolution MS scan was performed using the Orbitrap to select the 5 most intense ions prior to MS/MS analysis using the Ion trap. The raw mass spectral data was analysed using Bioworks Browser 3.3.1 SP1, a proteomics analysis platform. MS/MS spectra were sequence database searched using the algorithm TurboSEQUEST. The MS/MS spectra were searched against a redundant Human Swissprot database. The following search parameters were used: precursor-ion mass tolerance of 100ppm, fragment ion tolerance of 1.0 Da with methionine oxidation and cysteine carboxymidomethylation specified as differential modifications and a maximum of 2 missed cleavage sites allowed.

**Specificity of the anti-LRG antibodies**

Specific LRG antibodies used for ELISA (IBL) include; anti-Human LRG (162) Rabbit IgG Affinity Purified capture antibody and an HRP conjugated Anti-Human LRG (329) Rabbit IgG Fab’ Affinity Purified antibody. The specificity of the ELISA is 100% for Human LRG, and ≤0.01% for Human Aβ (1-42), Human sAPPβ (W), Human sAPPα.
Serum Biomarker Assessment

BNP was quantified using a Triage meter BNP assay (Biosite Inc.). Serum levels of LRG were quantified using a Human LRG Assay Kit (IBL) according to the manufacturer’s instructions. The sensitivity (lower detection limit) of the assay was 0.17ng/ml. The upper detection limit of this assay was 100ng/ml. The inflammatory cytokines IL-6 and tumor necrosis factor alpha (TNFα) were quantified in the CS serum using an ultra-sensitive immunoassay with electrochemiluminescence detection (Meso Scale Discovery) as instructed by the manufacturer. The sensitivity of these assays was 0.7 and 0.3 pg/ml, respectively. Upper detection limit of this assay was 40,000 pg/ml. The intra-assay coefficients of variations for LRG, IL-6 and TNFα were ≤5%, ≤6%, ≤8%, respectively. For LRG ELISAs, peripheral serum samples were ran on plates from the same Lot. Number. Analysis of peripheral serums were ran together on the same day. CS serum samples were also analysed together on the same day. Serum samples analysed for IL-6 and TNFα levels were assayed on the one plate at the same time.
Supplemental Figures and Figure Legends

Supplemental Figure 1

**Supplemental Figure 1.** Differential expression of leucine-rich alpha-2-glycoprotein (LRG) in asymptomatic hypertensive patients. Examples of two-dimensional difference gel electrophoresis (2D-DIGE) gel images highlighting the differentially expressed LRG protein variants spot ID: 0416 (1) and spot ID: 0442 (2) from low and high BNP sera. The bar graph illustrates a significant 2 fold increase in LRG 3D spot volumes when spots are pooled from the 11 patients for the two differentially expressed LRG variants.
Supplemental Figure 2. Relationship between serum levels of leucine-rich alpha-2-glycoprotein (LRG) and the degree of coronary stenosis. LRG levels were not associated with the extent of coronary disease within the subset undergoing angiography (n=40), p=0.68.

Supplemental Figure 3. Correlation between leucine-rich alpha-2-glycoprotein (LRG) and left ventricular mass index (LVMI). AH, asymptomatic hypertension; LVDD, asymptomatic left ventricular diastolic dysfunction; DHF, diastolic heart failure; SHF, systolic heart failure.
Supplemental References