Left Ventricular Dysfunction and CXCR3 Ligands in Hypertension: From Animal Experiments to a Population-Based Pilot Study

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Abstract

Detecting left ventricular (LV) dysfunction at an early stage is key in addressing the heart failure epidemic. In proteome profiling experiments in mice subjected either to aortic banding or sham, the circulating CXCR3 ligands monokine induced by interferon-γ (MIG) and interferon-γ inducible protein 10 (IP10) were 5 to 40 fold up-regulated at eight weeks. We assessed the diagnostic value of circulating NT-pro BNP and CXCR3 ligands (MIG, IP10, Interferon-inducible T-cell alpha chemo-attractant [I–TAC]) in patients with hypertension (>140/90 mm Hg) associated with subclinical (n = 19) or symptomatic (n = 16) diastolic LV dysfunction on echocardiography and healthy controls. NT–pro BNP, MIG, IP10, I–TAC all increased (p < 0.014) across the categories of worsening left ventricular dysfunction. In patients with symptomatic disease, MIG, IP10, and I–TAC increased 210% (p = 0.015), 140% (p = 0.007) and 120% (p = 0.035) more than NT-pro BNP. The optimal discrimination limits, obtained by maximizing Youden's index were 246 pmol/L, 65 pg/mL, 93 pg/mL, and 24 pg/mL, respectively. The odds ratios associated with the four biomarkers were significant (p < 0.010), ranging from 4.00 for IP10 to 9.69 for MIG. With adjustment for NT–pro BNP, the CXCR3 ligands retained significance (p ≤ 0.028). Adding optimized thresholds for the CXCR3 ligands to NT–pro BNP enhanced (p ≤ 0.014) the integrated discrimination improvement and the net reclassification improvement. In conclusion, congruent with the concept that inflammation plays a key role in the pathogenesis of LV dysfunction, MIG, IP10 and I–TAC add diagnostic accuracy over and beyond NT–pro BNP.
Introduction

Heart failure is a major public health problem affecting more than more than 23 million patients worldwide [1]. Increased longevity [2] and the recently improved survival after first diagnosis [1] explain why the prevalence of heart failure will continue rising by 50% over the next 10–15 years. The associated disability entails high health care costs exceeding those of cancer [2]. After the initial diagnosis, the estimated mortality rate is 25–28% at 1 year and 48–65% at 5 years [1]. Heart failure is a progressive condition, starting with the mere presence of risk factors, such as hypertension or diabetes (stage A) [3]. The next step includes asymptomatic changes in cardiac structure and function, as exemplified by left ventricular hypertrophy or impaired relaxation (stage B) [3]. Finally patients progress to clinically overt heart failure (stages C and D), disability, and death [3].

Screening tools that can identify heart failure patients early during the long asymptomatic course of the disease are of crucial importance in addressing the heart failure epidemic. Such tools not only allow timely intervention, but also help in characterizing the underlying molecular mechanisms and better targeting interventions. Circulating [4] or urinary [5] biomarkers reflecting inflammation [6], oxidative stress [7], cardiomyocyte injury [8], or left ventricular remodeling [5,9] are easily accessible and allow frequent re-evaluation. Inflammation is the driving force in causing myocardial damage in a wide variety of conditions, cardiac damage associated with myocardial ischemia, myocardial infarction, and viral myocarditis [10,11].

Our current study aimed to identify novel inflammatory biomarkers for left ventricular dysfunction by performing a screen of 40 inflammatory biomarkers, including CXCR3 ligands [12], in serum obtained from mice subjected to aortic banding. Next, we assessed biomarkers identified in the animal experiments as screening tools in a case-control study nested within the FLEMish study on Environment, Genes and Health Outcome (FLEMENGHO) [13,14].

Methods

Experimental studies in mice

The Animal Care and Use Committee of Maastricht University approved the experimental studies in male C57BL/6J mice (Harlan Laboratories, Horst, The Netherlands). As described in detail elsewhere [15], we induced pressure overload in by aortic banding. Control animals underwent the same surgical procedure without the actual tightening of the ligature (sham). We assessed left ventricular contractility at 4 or 8 weeks after the intervention under urethane anaesthesia (+dP/dt [16,17]), just before the animals (n = 30) were sacrificed by exsanguination through blood sampling from the abdominal aorta. It was then allowed to clot for 4 hours at 4°C before centrifugation at 2000 g for 10 minutes. Next, serum was removed, immediately aliquoted, and stored at −80°C.

The profile of 40 inflammatory mediators was determined, using the Proteome Profiler Mouse Cytokine Array Kit, Panel A (R&D systems, Abingdon, United Kingdom) with improved fluorescence detection, as described previously. Membranes were incubated with 1 mL of pooled serum obtained from 4 to 6 animals per group. A total volume of 1000 μL (maximum possible volume) was applied to the membrane. To determine 40 extracellular cytokines in a semi-quantitative manner, we followed the manufacturer’s instructions available online at http://www.rndsystems.com/product_detail_objectname_ProteomeProfilerArray.aspx. We modified this protocol after step 9 to acquire semi-quantitative measurements by use of a fluorescence read-out [18].

Nested case-control study

FLEMENGHO is a large-scale family-based study on the genetic epidemiology of cardiovascular phenotypes, for which recruitment started in 1985 and continued through 2008 [13,14].
The Ethics Committee of the University of Leuven approved the FLEMENGHO study. Participants gave written informed consent. As reported previously[5], we selected 35 cases and 35 controls from a cohort [19], who from 2005 until 2010 underwent echocardiography including tissue Doppler imaging by means of the Vivid7 Pro scanner (GE Vingmed, Horten, Norway) interfaced with a 2.5- to 3.5-MHz phased-array probe. We followed the recommendations of the American Society of Echocardiography [20] for data acquisition and the offline analysis. Cases were hypertensive patients with either asymptomatic (n = 19) or symptomatic (n = 16) diastolic left ventricular dysfunction and controls were normotensive healthy controls [5]. The diagnosis of left ventricular dysfunction relied on earlier published age-specific echocardiographic criteria for impaired relaxation and increased left ventricular filling pressure [5,19]. Participants reported cardiovascular, dyspnea, and respiratory symptoms by completing the London School of Hygiene questionnaires [21] and intake of medications and lifestyle characteristics via an observer-administered standardized questionnaire. Blood pressure was measured twice in the supine position, after echocardiography, using the validated [22] OMRON 705IT device (OMRON Healthcare Europe BV, Nieuwegein, The Netherlands). Hypertension was a blood pressure of at least 140 mm Hg systolic or 90 mm Hg diastolic or use of antihypertensive drugs. Body mass index was weight in kilogram divided by height in meter squared.

With participants fasting for at least 6 hours, venous blood samples were drawn for measurement of serum cholesterol, plasma glucose, and biomarkers. Diabetes was identified by the use of antidiabetic drugs, a plasma glucose of at least 7.0 mmol/L, a self-reported diagnosis, or diabetes documented in practice or hospital records [23]. Blinded to the clinical data, we measured the levels of circulating inflammatory biomarkers by enzyme linked immunosorbent assay (ELISA), using the Human CXCL9/MIG Quantikine ELISA Kit, the Human CXCL10/IP10 Quantikine ELISA Kit and the Human CXCL11/1–TAC Quantikine ELISA (R&D Systems, Minneapolis MN). We also determined plasma N–terminal pro–atrial natriuretic peptide (NT–pro BNP) by a competitive enzyme immunoassay designed for research only (Biomedica Gruppe, Vienna, Austria) [24]. Standard NT–proBNP levels range 0 to 1000 pmol/L (median, 208 pmol/L; 95th, percentile 300 pmol/L) [24]. Because of insufficient plasma sample volume, the maximum number of cases and controls analyzed in our current manuscript amounted to 31 and 32, respectively; the minimum numbers were 29 cases and 29 controls.

We compared means by a t-test or ANOVA and proportions by Fisher’s exact test, using SAS software, version 9.3 (SAS Institute, Cary, NC, USA). Significance was a two-tailed \( \alpha \)–level of 0.05 or less. We normalized the distributions of the circulating biomarkers by logarithmic transformation. We modeled the odds of being a case in relation to the circulating biomarkers by logistic regression. We used mixed models to test the null hypothesis that increases in new biomarkers in patients with left ventricular dysfunction were identical to that observed for NT–pro BNP. We determined optimal discrimination limits for biomarkers by maximizing the Youden’s index (sensitivity plus specificity minus 1) [25]. We assessed the diagnostic accuracy of the inflammatory biomarkers, using the integrated discrimination improvement (IDI) and the net reclassification improvement (NRI) [26]. IDI is the difference between the discrimination slopes of basic models and basic models extended with a predictor variable. The discrimination slope is the difference in predicted probabilities (%) between cases and controls. NRI is the sum of the percentages of subjects reclassified correctly into cases and controls.

Results

Experimental studies in mice

We assessed cardiac hemodynamics in mice subjected to transverse aortic banding and in sham operated controls before and after dobutamine infusion. Baseline +dP/dt before infusion
of dobutamine was similar in banded and control animals, averaging (SE) 129 ± 7 vs. 155 ± 3 mmHg/s at 4 weeks, and 149 ± 20 vs. 156 ± 13 mmHg/s at 8 weeks. However, upon maximal stimulation with dobutamine (6–7 ng/g/min), the increase in +dP/dt was attenuated (p < 0.05) in banded compared with control mice, averaging 239 ± 19 vs. 298 ± 17 mmHg/s at 4 weeks and 215 ± 19 vs. 296 ± 8 mmHg/s at 8 weeks.

In the proteome profiling experiments, 5 of 40 cytokines stood out as being at least 2–fold up-regulated (Fig 1). They included in order of the degree of up-regulation: monokine induced by interferon-γ (MIG [CXCL9]), interferon-γ inducible protein 10 (IP10 [CXCL10]), murine macrophage inflammatory protein-2, interleukin 16 (IL–16), and soluble intercellular adhesion molecule 1 (sICAM–1). MIG and IP-10 are ligands of the CXCR3 receptors, which are predominantly expressed by T-lymphocytes and natural killer cells [27]. CXCR3 receptors also bind Interferon-inducible T-cell alpha chemo-attractant (I–TAC [CXCL11]) [27]. Based on our current experiments in mice and the available literature [12,27], we carried MIG, IP10 and I–TAC further in the case-control study nested in the FLEMENGHO echocardiographic studies.

**Nested case-control study**

Compared to healthy controls (Table 1), patients with subclinical left ventricular dysfunction, were older and had higher body mass index, blood pressure, and were more frequently on treatment with diuretics and β-blockers (p ≤ 0.029). Compared to patients with subclinical diastolic left ventricular dysfunction (Table 1), symptomatic patients were older, had higher plasma glucose, had a higher prevalence of coronary heart disease, and were more frequently on treatment with vasodilators (p ≤ 0.032). The echocardiographic measurements of the healthy controls and of the patients with subclinical or symptomatic left ventricular dysfunction appear in S1 Table.

Circulating levels of MIG, IP10, I–TAC and NT–pro BNP all increased (p ≤ 0.014) across the categories of worsening left ventricular dysfunction (Table 2). Compared with healthy controls, circulating levels of the four biomarkers were similar in patients with subclinical left
ventricular dysfunction (p ≥ 0.14), whereas they were significantly elevated (p ≤ 0.011) in symptomatic patients (Table 2). In patients with symptomatic disease, MIG, IP10, and I–TAC increased 210% (p = 0.015), 140% (p = 0.007) and 120% (p = 0.035) more than NT–pro BNP (Fig 2). By maximizing Youden’s index, the optimal discrimination limits of MIG, IP10, I–TAC and NT–pro BNP respectively were 65 g/mL, 93 pg/mL, 24 pg/mL, and 246 pmol/L (S2 Table). Table 3 shows the odds ratios for having left ventricular dysfunction in relation to the biomarkers. In unadjusted analyses, the odds ratios associated with the four biomarkers were all significant (p ≤ 0.010), ranging from 4.00 for IP10 to 9.69 for MIG. With adjustment for NT–pro BNP, the CXCR3 ligands retained significance (p ≤ 0.028). The odds ratios were 6.95 for MIG, 3.74 for IP10 and 12.3 for I–TAC. Adding the optimized thresholds for the CXCR3 ligands to basic models including NT–pro BNP alone or in combination with age and body mass index (Table 4) significantly improved IDI (p ≤ 0.038) and NRI (p ≤ 0.005) except for IP10 in models also including age and body mass index (p = 0.42). Sensitivity analyses, in which we computed odds ratios (S3 Table), IDI and NRI (S4 Table), with the biomarkers expressed on a continuous scale, were largely confirmatory, although for IDI significance was only reached for the three CXCR3 ligands combined added to a basic model only including NT–pro BNP.

| Characteristics | Control (n = 32) | Subclinical LV dysfunction (n = 17) | Symptomatic LV dysfunction (n = 14) |
|-----------------|-----------------|-----------------------------------|-----------------------------------|
| Number of subjects |                 |                                   |                                   |
| Women (%)       | 14 (43.7)       | 9 (52.9)                          | 8 (57.1)                          |
| Smoker (%)      | 11 (34.4)       | 4 (23.5)                          | 2 (14.3)                          |
| Drinker (%)     | 28 (87.5)       | 12 (70.6)                         | 7 (50.0)                          |
| Coronary heart disease (%) | 1 (3.1) | 1 (5.9)                            | 6 (42.9)*                         |
| Hypertensive drug treatment | 0 | 12 (70.6)†                          | 10 (71.4)                         |
| Diuretics (%)   | 0               | 7 (41.2)‡                         | 7 (50.0)                           |
| β-blockers (%)  | 0               | 9 (52.9)‡                         | 5 (35.7)                           |
| RAS inhibitors (%) | 0            | 2 (11.8)                            | 5 (35.7)                           |
| Vasodilators (%) | 0             | 0                                 | 4 (28.6)*                         |
| Mean characteristic (±SD) |             |                                   |                                   |
| Age (year)      | 62.8 ± 6.1      | 67.4 ± 7.6*                       | 73.9 ± 8.3*                       |
| Body mass index (kg/m²) | 25.0 ± 2.4 | 29.8 ± 4.3‡                       | 30.2 ± 4.2                        |
| Blood pressure  |                 |                                   |                                   |
| Systolic (mm Hg) | 122.3 ± 9.0     | 150.9 ± 9.6‡                      | 147.8 ± 21.4                      |
| Diastolic (mm Hg) | 74.6 ± 4.9     | 81.7 ± 10.5*                      | 77.8 ± 10.9                       |
| Mean (mm Hg)    | 90.5 ± 4.5      | 104.8 ± 8.6‡                      | 101.1 ± 11.7                      |
| Heart rate (beats per minute) | 62.5 ± 7.1 | 60.9 ± 8.8                         | 64.6 ± 16.3                       |
| Serum cholesterol (mmol/L) | 5.48 ± 0.75 | 5.60 ± 1.08                        | 5.41 ± 1.19                       |
| Blood glucose (mmol/L) | 4.90 ± 0.39 | 4.74 ± 0.54                        | 5.41 ± 0.70†                      |

LV indicates left ventricle. Mean arterial pressure was diastolic blood pressure plus one third of the difference between systolic and diastolic blood pressure. Inhibitors of the renin-angiotensin system included angiotensin-converting enzyme inhibitors and angiotensin type–1 receptor blockers. Vasodilators were calcium channel blockers and α-blockers. P–values for trend across categories were significant (p ≤ 0.025) except for the proportion of women and smokers and the mean values of heart rate and serum cholesterol (p ≥ 0.39). Significance of the difference with left adjacent group:

* p ≤ 0.05
† p ≤ 0.01; and
‡ p ≤ 0.001.

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Table 1. Clinical Characteristics of Controls and Cases.
The main finding of our study was that by performing an unbiased screen for inflammatory biomarkers in a mouse model of heart failure, we identified a cluster of three related chemokines that were present in elevated concentrations in the plasma of patients with symptomatic diastolic left ventricular dysfunction. These three CXCR3 ligands enhanced diagnostic accuracy over and beyond NT-pro BNP, as exemplified by the integrated discrimination improvement and the net reclassification improvement.

Heart failure is a major and growing public health problem with a complex etiology [28]. The diagnosis of heart failure, particularly in the early stage when clinical symptoms are still mild or even absent, remains a challenge. Several circulating biomarkers are currently considered in the diagnosis and management of heart failure. BNP and NT-proBNP are natriuretic peptides, which are released from the myocardium upon excessive stretch. Their circulating levels are elevated in heart failure patients and useful for confirmation of the diagnosis in

| Biomarker | Control | Subclinical LV dysfunction | Symptomatic LV dysfunction | p for trend |
|-----------|---------|----------------------------|---------------------------|------------|
| Monokine induced by interferon-γ (MIG [CXCL9]) | n° of participants: 32 | 17 | 14 | 0.014 |
| Level (pg/mL): 40.3 (29.9–54.2) | 56.7 (28.6–98.0) | 111.0 (39.6–332.5) |
| Interferon-γ inducible protein 10 (IP10 [CXCL10]) | n° of participants: 32 | 17 | 14 | 0.001 |
| Level (pg/mL): 80.7 (55.0–113.7) | 105.1 (63.4–140.5) | 148.2 (81.4–213.2) |
| Interferon-inducible T-cell alpha chemo-attractant (I–TAC [CXCL11]) | n° of participants: 31 | 13 | 13 | 0.008 |
| Level (pg/mL): 14.9 (7.80–22.4) | 22.4 (7.80–35.2) | 32.3 (23.2–56.9) |
| N–terminal pro–brain natriuretic peptide (NT–pro BNP) | n° of participants: 29 | 16 | 13 | 0.005 |
| Level (pmol/L): 202.1 (150.6–305.9) | 284.3 (156.0–544.6) | 380.7 (258.8–663.4) |

LV indicates left ventricle. Biomarker levels are geometric means (interquartile ranges).

Significance with healthy controls:
* p < 0.05
† p < 0.01; and
‡ p < 0.001.

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### Discussion

The main finding of our study was that by performing an unbiased screen for inflammatory biomarkers in a mouse model of heart failure, we identified a cluster of three related chemokines that were present in elevated concentrations in the plasma of patients with symptomatic diastolic left ventricular dysfunction. These three CXCR3 ligands enhanced diagnostic accuracy over and beyond NT–pro BNP, as exemplified by the integrated discrimination improvement and the net reclassification improvement.

Heart failure is a major and growing public health problem with a complex etiology [28]. The diagnosis of heart failure, particularly in the early stage when clinical symptoms are still mild or even absent, remains a challenge. Several circulating biomarkers are currently considered in the diagnosis and management of heart failure. BNP and NT–proBNP are natriuretic peptides, which are released from the myocardium upon excessive stretch. Their circulating levels are elevated in heart failure patients and useful for confirmation of the diagnosis in

| Biomarker | Unadjusted odds ratio | p | Odds ratio adjusted for NT–pro BNP | p |
|-----------|-----------------------|---|-----------------------------------|---|
| MIG (≥65 pg/mL) | 9.69 (2.73 to 34.4) | 0.0004 | 6.95 (1.81 to 26.6) | 0.005 |
| IP10 (≥93 pg/mL) | 4.00 (1.40 to 11.4) | 0.010 | 3.74 (1.15 to 12.1) | 0.028 |
| I–TAC (≥24 pg/mL) | 7.09 (2.07 to 24.3) | 0.002 | 12.3 (2.40 to 63.3) | 0.003 |
| NT–pro BNP (≥246 pmol/L) | 6.98 (2.19 to 22.2) | 0.001 | … | … |

Abbreviations of the biomarkers are spelled out in Table 2. Odds ratios (95% confidence intervals) express the risk associated with a biomarker concentration above the optimized threshold.

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patients with dyspnea [3]. Troponin T and I are markers of myocardial damage when cardiac ischemia or myocardial infarction occurs. After the introduction of a high-sensitive assay, the application of troponin T and I extended to myocardial injury as a consequence of advanced heart failure [4]. Wang and colleagues measured 10 biomarkers, including B-type natriuretic peptide (BNP) and NT–pro BNP in 3209 participants attending a routine examination cycle of the Framingham Heart Study [29]. During a median follow-up of 7.4 year, 207 participants died and 169 had a first major cardiovascular event. In Cox proportional-hazards models with adjustments applied for conventional risk factors, BNP, but not NT–pro BNP was a strong predictor. The Framingham investigators also combined circulating biomarkers into multimarker scores [4]. However, the addition of multimarker scores to conventional risk factors resulted in only small enhancements in risk stratification. As reviewed elsewhere [30], these Framingham findings [4] exemplify the need to develop new biomarkers with higher diagnostic and predictive value when used alone or in combination.

In the past decade, the research community made large efforts to identify novel biomarkers that would allow a better characterization of the disease process underlying left ventricular
dysfunction and an improved stratification of prognosis and success of pharmacotherapy.

C-reactive protein is an independent predictor of adverse outcomes in patients with acute or chronic heart failure [28]. Other biomarkers that established a link between heart failure and inflammation include tumor necrosis factor-alpha [31], and the pro-inflammatory (e.g. interleukin–6 [32]) and anti-inflammatory (interleukin–10 [33]) cytokines.

Inflammatory biomarkers probably contain valuable information with respect to the molecular mechanisms initiating left ventricular dysfunction and its progression to symptomatic heart failure. In the present study, we focused on the circulating CXCR3 ligands, MIG, IP10 and I–TAC. These CXCR3-agonistic cytokines are involved in autoimmune diseases, such as rheumatoid arthritis and systemic lupus erythematosus [12]. They play a role in recruiting immune cells to the sites of inflammation. Hypertensive patients also have elevated levels of circulating MIG, IP10 and I–TAC [34–37]. Whether this is causal or just reflecting target organ damage remains currently unknown. In this context it should be noted that in our patients with left ventricular dysfunction, the average blood pressure was elevated, while the majority were receiving antihypertensive drugs. However, all CXCR3 ligands showed higher average levels in patients with symptomatic left ventricular dysfunction, despite the fact that the blood pressure tended to be lower in these patients. These observations suggest that the elevated levels of MIG and I–TAC are not solely the result of an increased blood pressure, but also correlate with the degree of left ventricular dysfunction.

An important prerequisite for a biomarker is its involvement in the disease. Is it just an innocent bystander or a causal component of the pathological process [4]? Inflammatory mediators drive cardiac remodeling in response to ischemia, myocarditis and grafting [38,39]. However, hypertension was probably the trigger of left ventricular dysfunction in our participants. Although hemodynamic stress can induce an inflammatory state in the myocardium, the inflammatory triggers are not entirely certain [10]. The CXCR3 ligands MIG, IP10 and I–TAC are involved in the recruitment of immune cells, particularly T-lymphocytes, into sites of inflammation [12,40]. Augmented infiltration of CD4+ T-lymphocytes is associated with a rapid deterioration of the ejection function in a rat model of spontaneous hypertension [41].

Table 4. Net Reclassification Improvement and Integrated Discrimination Improvement by Adding Optimized Thresholds of the CXCR3 Ligands to the Basic Models.

| Variables in basic models biomarkers | Integrated discrimination improvement | Net reclassification improvement |
|-------------------------------------|--------------------------------------|---------------------------------|
|                                     | Δ% (95% confidence interval) p        | Δ% (95% confidence interval) p   |
| NT-pro BNP                          |                                      |                                 |
| MIG                                 | 12.9 (4.01 to 21.8) 0.004            | 89.7 (45.9 to 133.4) <0.0001    |
| IP10                                | 7.51 (1.55 to 13.5) 0.014            | 69.0 (20.7 to 117.3) 0.005      |
| I–TAC                               | 17.8 (7.67 to 28.0) 0.006            | 84.3 (36.5 to 132.0) 0.0005     |
| MIG + IP10 + I–TAC                  | 22.8 (10.4 to 35.2) 0.0003           | 124.2 (82.0 to 166.6) <0.0001   |
| NT-pro BNP, age and body mass index |                                      |                                 |
| MIG                                 | 5.45 (0.29 to 10.6) 0.038            | 110.3 (68.3 to 152.4) <0.0001   |
| IP10                                | 0.63 (–0.90 to 2.16) 0.42            | 69.0 (20.7 to 117.3) 0.005      |
| I–TAC                               | 9.64 (1.33 to 18.0) 0.023            | 154.6 (120.3 to 188.8) <0.0001  |
| MIG + IP10 + I–TAC                  | 18.8 (9.62 to 28.0) <0.0001          | 192.9 (179.1 to 206.6) <0.0001  |

Abbreviations of the biomarkers are spelled out in Table 2. The net reclassification improvement is the sum of the percentages of subjects reclassified correctly in cases and controls. The integrated discrimination improvement is the difference between the discrimination slopes of the extended and basic models. The discrimination slope is the difference in predicted probabilities between cases and controls. Cases were patients with subclinical or symptomatic diastolic left ventricular dysfunction. Controls were healthy people.

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Although the precise roles of the different T-lymphocyte subsets in the inflammatory response to cardiac hemodynamic stress have not yet been fully clarified, our current data support the involvement of CXCR3 ligands in left ventricular dysfunction.

One of the limitations of the present study is that due to the experimental setup we could not identify the source of the CXCR3 ligands that were elevated in the circulation of patients with left ventricular dysfunction. Inducible expression of IP10 has been demonstrated in monocytes, endothelial cells and fibroblasts [42,43] and regulates the reparative response in an experimental model of myocardial infarction [44] To our knowledge, no previous study reported on the functional effects of CXCR3 agonists on the response of the heart to hemodynamic overload. Additional work in animal models will be needed to resolve this question. Other issues that must be addressed after this pilot study, is independent confirmation of the diagnostic accuracy of circulating CXCR3 ligands in patients with early stages of left ventricular dysfunction and validation of these new markers in terms of outcome and responses to treatment. In conclusion, left ventricular dysfunction is associated with elevated circulating levels of the CXCR3 ligands MIG, IP10 and I–TAC. Adding the levels of these chemokines to the risk prediction model provided a significant net reclassification and integrated discrimination improvement. However, further experimental, clinical and epidemiological studies are required to substantiate the findings from our pilot study.

Supporting Information
S1 Table. Echocardiographic characteristics in cases and controls. (DOCX)
S2 Table. Optimized diagnostic thresholds for the circulating biomarkers in relation to left ventricular dysfunction. (DOCX)
S3 Table. Odds ratios expressing the risk of left ventricular dysfunction in relation to biomarkers analyzed as continuous variables. (DOCX)
S4 Table. Net reclassification improvement and integrated discrimination improvement by adding CXCR3 ligands as continuous variables to basic models. (DOCX)

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Author Contributions
Conceived and designed the experiments: JAS WMB HAS-B. Performed the experiments: Y-MG RA. Analyzed the data: Y-MG RA. Contributed reagents/materials/analysis tools: LT. Wrote the paper: JAS Y-MG RA WMB. Reviewed the manuscript: LT.
References

1. Dunlay SM, Roger VL (2014) Understanding the epidemic of heart failure: past, present, and future. Curr Heart Fail Rep 11:404–415. doi: 10.1007/s11897-014-0220-x PMID: 25182014

2. Rosamond W, Flegal K, Furie K, Go A, Greenland K, Haase N, et al. (2008) Heart disease and stroke statistics—2008 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Circulation 117:e25–e146. PMID: 18086926

3. McMurray JJ, Adamopoulos S, Anker SD, Auricchio A, Böhm M, Dickstein K, et al. (2012) ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure 2012: The Task Force for the Diagnosis and Treatment of Acute and Chronic Heart Failure of the European Society of Cardiology. Developed in collaboration with the Heart Failure Association (HFA) of the ESC. Eur Heart J 33:1787–1847. doi: 10.1093/eurheartj/ehs104 PMID: 22611136

4. van Kimmenade RR, Januzzi JL Jr (2012) Emerging biomarkers in heart failure. Clin Chem 58:127–138. doi: 10.1373/clinchem.2011.165720 PMID: 22086968

5. Kuznetsova T, Mischak H, Mullen W, Staessen JA (2012) Urinary proteome analysis in hypertensive patients with left ventricular diastolic dysfunction. Eur Heart J 33:3242–3250. doi: 10.1093/eurheartj/ehs185 PMID: 22789915

6. Jarr KU, Neelmes M, Katus HA, Chorianopoulos E (2014) Inter- not intraindividual differences in sTWEAK levels predict functional deterioration and mortality in patients with diluted cardiomyopathy. Mediators Inflamm 2014:756482. doi: 10.1155/2014/756482 PMID: 25089077

7. Palmieri B, Splendoro V (2007) Oxidative stress tests: overview on reliability and use. Part I. Eur Rev Med Pharmacol Sci 11:309–3. PMID: 18074940

8. Parissis JT, Papadakis J, Kadoglou NP, Varounis C, Psarogiannakopoulos P, Rafouli-Stergiou P, et al. (2013) Prognostic value of high sensitivity troponin T in patients with acutely decompensated heart failure and non-detectable conventional troponin T levels. Int J Cardiol 168:3609–3612. doi: 10.1016/j.ijcard.2013.05.056 PMID: 23711451

9. López B, González A, Díez J (2010) Circulating biomarkers of collagen metabolism in cardiac diseases. Circulation 121:1645–1654. doi: 10.1161/CIRCULATIONAHA.109.127774 PMID: 20385961

10. Coggins M, Rosenzweig A (2012) The fire within: Cardiac inflammatory signaling in health and disease. Circ Res 110:116–125. doi: 10.1161/CIRCRESAHA.111.243196 PMID: 22223209

11. Marchant DJ, Boyd JH, Lin DC, Granville DJ, Garmaroudi FS, McManus BM. (2012) Inflammation in myocardial diseases. Circ Res 110:126–144. doi: 10.1161/CIRCRESAHA.111.243170 PMID: 22223210

12. Lacotte S, Brun S, Muller S, Dumortier H (2009) CXCR3, inflammation, and autoimmune diseases. Ann N Y Acad Sci 1173:310–317. doi: 10.1111/j.1749-6632.2009.04813.x PMID: 19795167

13. Staessen JA, Wang JG, Brand E, Barlassina C, Birkenhäger WH, Herrmann SM, et al. (2001) Effects of three candidate genes on prevalence and incidence of hypertension in a Caucasian population. J Hypertens 19:1349–1358. PMID: 11518842

14. Li Y, Zagato L, Kuznetsova T, Tripodi G, Zerbini G, Richart T, et al. (2007) Angiotensin-converting enzyme I/D and α-adducin Gγ460Trp polymorphisms. From angiotensin-converting enzyme activity to cardiovascular outcome. Hypertension 49:1291–1297. PMID: 17452507

15. van de Schans VA, van den Borne SW, Strzelecka AE, Janssen BJ, van der Velden JL, Langen RC, et al. (2007) Interruption of wnt signaling attenuates the onset of pressure overload-induced cardiac hypertrophy. Hypertension 49:473–480. PMID: 17210832

16. De Celle T, Cleutjens JP, Blankesteijn WM, Debets JJ, Smits JF, Janssen BJ (2004) Long-term structural and functional consequences of cardiac ischaemia-reperfusion injury in vivo in mice. Exp Physiol 89:605–615. PMID: 15258119

17. Heijnen BF, Peikmans LP, Danser AJ, Garrelks IM, Mullins JJ, De Mey JG, et al. (2014) Cardiac remodeling during and after renin-angiotensin system stimulation in CYP1a1-Ren2 transgenic rats. J Renin Angiotensin Aldosterone Syst 15:69–81. doi: 10.1177/1470320314505507 PMID: 23462119

18. Altara R, Manca M, Hessel MH, Janssen BJ, Struijker-Boudier HA, Hermans RJ, et al. (2014) Improving membrane based multiplex immunoassays for semi-quantitative detection of multiple cytokines in a single sample. BMC Biotechnol 14:63. doi: 10.1186/1472-6750-14-63 PMID: 25022797

19. Kuznetsova T, Herbots L, López B, Jin Y, Richart T, Thijs L, et al. (2009) Prevalence of left ventricular diastolic dysfunction in a general population. Circ Heart Fail 2:105–112. doi: 10.1161/CIRCHEARTFAILURE.108.822627 PMID: 19808325

20. Gottdiener JS, Bednarz J, Devereux R, Gardin J, Klein A, Manning WJ, et al. (2004) American Society of Echocardiography recommendations for use of echocardiography in clinical trials. A report from the American Society of Echocardiography’s Guidelines and Standard Committee and the Task Force on Echocardiography in Clinical Trials. J Am Soc Echocardiogr 17:1086–1119. PMID: 15452479
21. Rose GA, Blackburn H (1968) Cardiovascular survey methods. *Monogr Ser World Health Organ* 56:1–188.

22. El Assaad MA, Topouchian JA, Asmar RG (2003) Evaluation of two devices for self-measurement of blood pressure according to the international protocol: the Omron M5-I and the Omron 705IT. *Blood Press Monit* 8:127–133. PMID: 12900590

23. Expert Committee on the Diagnosis and Classification of Diabetes Mellitus (2006) Report of the expert committee on the diagnosis and classification of diabetes mellitus. *Diabetes Care* 26 Suppl 1:S5–S20.

24. Mueller T, Gegenhuber A, Poelz W, Haltmayer M (2003) Comparison of the Biomedica NT-pro BNP enzyme immunoassay and the Roche NT-pro BNP chemiluminescence immunoassay: implication for the prediction of symptomatic and asymptomatic structural heart disease. *Clin Chem* 49:976–979. PMID: 12766002

25. Schisterman EF, Perkins NJ, Liu A, Bondell H (2005) Optimal cut-point and its corresponding Youden index to discriminate individuals using pooled blood samples. *Epidemiology* 16:73–81. PMID: 15613948

26. Pencina MJ, D’Agostino RB Sr., Steyerberg EW (2011) Extensions of net reclassification improvement calculations to measure usefulness of new biomarkers. *Stat Med* 30:11–21. doi: 10.1002/sim.4085 PMID: 21204120

27. Scholten DJ, Canals M, Wijtmans M, de Munnik S, Nguyen P, Verzijl D, et al. (2012) Characterization of a small-molecule agonist of the chemokine receptor CXCR3. *Br J Pharmacol* 166:898–911. doi: 10.1111/j.1476-5381.2011.01648.x PMID: 21883151

28. Braunwald E (2008) Biomarkers in heart failure. *N Engl J Med* 258:2148–2159.

29. Wang TJ, Gona P, Larson MG, Tofler GH, Levy D, Newton-Cheh C, et al. (2006) Multiple biomarkers for the prediction of first major cardiovascular events and death. *N Engl J Med* 355:2631–2639. PMID: 17182988

30. Gerszten RE, Wang TJ (2008) The search for new cardiovascular biomarkers. *Nature* 451:949–952. doi: 10.1038/nature06802 PMID: 18298185

31. Levine B, Kalman J, Mayer L, Fillit HM, Packer M (1990) Elevated circulating levels of tumor necrosis factor in severe chronic heart failure. *N Engl J Med* 323:236–241. PMID: 2195340

32. Bozkurt B, Mann DL, Deswal A (2010) Biomarkers of inflammation in heart failure. *Heart Fail Rev* 15:331–341. doi: 10.1007/s10741-009-9140-3 PMID: 19363700

33. Kaur K, Dhingra S, Slezak J, Sharma AK, Bajaj A, Singal PK. (2009) Biology of TNF alpha and IL-10, and their imbalance in heart failure. *Heart Fail Rev* 14:113–123. doi: 10.1007/s10741-008-9104-z PMID: 18712475

34. Antonelli A, Fallahi P, Rotondi M, Ferrari SM, Romagnani P, Ghiadoni L, et al. (2008) High serum levels of CXC chemokine ligand 10 in untreated essential hypertension. *J Hum Hypertens* 22:579–581. doi: 10.1038/jhh.2008.15 PMID: 18337756

35. Youn JC, Yu HT, Lim BJ, Koh MJ, Lee J, Chang DY, et al. (2013) Immunosenescent CD8+ T cells and C-X-C chemokine receptor type 3 chemokines are increased in human hypertension. *Hypertension* 62:126–133. doi: 10.1161/HYPERTENSIONAHA.113.00689 PMID: 23716586

36. Antonelli A, Fallahi P, Ferrari SM, Ghiadoni L, Virdis A, Mancusi C, et al. (2012) High serum levels of CXC (CXCL10) and CC (CCL2) chemokines in untreated essential hypertension. *Int J Immunopathol Pharmacol* 25:387–395. PMID: 22697070

37. Stumpf C, Auer C, Yilmaz A, Lewczuk P, Klinghammer L, Schneider M, et al. (2011) Serum levels of the Th1 chemoattractant interferon-gamma-inducible protein (IP) 10 are elevated in patients with essential hypertension. *Hypertens Res* 34:484–488. doi: 10.1038/hr.2010.258 PMID: 21228779

38. Frangogiannis NG (2012) Regulation of the inflammatory response in cardiac repair. *Circ Res* 110:159–173. doi: 10.1161/CIRCRESAHA.111.243162 PMID: 22223212

39. Hedayat M, Mahmoudi MJ, Rose NR, Rezaei N (2010) Proinflammatory cytokines in heart failure: Double-edged swords. *Heart Fail Rev* 15:543–562. doi: 10.1007/s10741-010-9168-4 PMID: 20405319

40. Groom JR, Luster AD (2011) CXCR3 ligands: redundant, collaborative and antagonistic functions. *Immunol Cell Biol* 89:207–215. doi: 10.1038/icb.2010.158 PMID: 21221121

41. Zandbergen HR, Sharma UC, Gupta S, Verjans JW, van den Borne S, Pokharel S, et al. (2009) Macrophage depletion in hypertensive rats accelerates development of cardiomyopathy. *J Cardiovasc Pharmacol Ther* 14:68–75. doi: 10.1177/1074242408329860 PMID: 19168432

42. Farber JM (1997) MIG and IP-10: CXC chemokines that target lymphocytes. *J Leukoc Biol* 61:246–257. PMID: 9060447

43. Luster AD, Ravetch JV (1987) Biochemical characterization of a gamma interferon-inducible cytokine (IP-10). *J Exp Med* 166:1084–1097. PMID: 2443596
44. Bujak M, Dobaczewski M, Gonzalez-Quesada C, Xia Y, Leucker T, Zymek P, et al. (2009) Induction of the CXC chemokine interferon-gamma-inducible protein 10 regulates the reparative response following myocardial infarction. Circ Res 105:973–983. doi: 10.1161/CIRCRESAHA.109.199471 PMID: 19797174