Chromogranin B in Heart Failure
A Putative Cardiac Biomarker Expressed in the Failing Myocardium

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Background—Chromogranin B (CgB) is a member of the granin protein family. Because CgB is often colocalized with chromogranin A (CgA), a recently discovered cardiac biomarker, we hypothesized that CgB is regulated during heart failure (HF) development.

Methods and Results—CgB regulation was investigated in patients with chronic HF and in a post–myocardial infarction HF mouse model. Animals were phenotypically characterized by echocardiography and euthanized 1 week after myocardial infarction. CgB mRNA levels were 5.2-fold increased in the noninfarcted part of the left ventricle of HF animals compared with sham-operated animals (P<0.001). CgB mRNA level in HF animals correlated closely with animal lung weight (r=0.74, P=0.04) but not with CgA mRNA levels (r=0.20, P=0.61). CgB protein levels were markedly increased in both the noninfarcted (110%) and the infarcted part of the left ventricle (70%) but unaltered in other tissues investigated. Myocardial CgB immunoreactivity was confined to cardiomyocytes. Norepinephrine, angiotensin II, and transforming growth factor-β increased CgB gene expression in cardiomyocytes. Circulating CgB levels were increased in HF animals (median levels in HF animals versus sham, 1.23 [interquartile range, 1.03 to 1.93] versus 0.98 [0.90 to 1.04] nmol/L; P=0.003) and in HF patients (HF patients versus control, 1.66 [1.48 to 1.85] versus 1.47 [1.39 to 1.58] nmol/L; P=0.007), with levels increasing in proportion to New York Heart Association functional class (P=0.03 for trend). Circulating CgB levels were only modestly correlated with CgA (r=0.31, P=0.009) and B-type natriuretic peptide levels (r=0.27, P=0.014).

Conclusions—CgB production is increased and regulated in proportion to disease severity in the left ventricle and circulation during HF development. (Circ Heart Fail. 2010;3:503-511.)

Key Words: heart failure • molecular biology • biological markers • chromogranin B

Advances in treatment of acute coronary syndromes have reduced short-term mortality following acute myocardial infarction (MI), but at the cost of a higher incidence of post-MI heart failure (HF).1 Improved pathophysiological understanding of HF and better tools for risk prediction and diagnostic purposes are therefore needed.2 The cardiac biomarkers used routinely in clinical practice today are proteins specific to the diseased myocardium, either released during cell necrosis (cardiac specific troponins)3 or secreted secondary to cardiomyocyte strain (B-type natriuretic peptide, BNP).4 However, there is still a need for markers reflecting other pathophysiological processes in heart disease.5 Thus, identifying new proteins regulated in the failing myocardium may potentially improve both the understanding of HF development and lead to the discovery of novel cardiac biomarkers.

Clinical Perspective on p 511
Chromogranin B (CgB) is a 50-kDa protein belonging to a group of acidic proteins called the granin protein family.6 The most extensively studied and well-known member of the granin family is chromogranin A (CgA), a protein currently used clinically as a biomarker in patients with neuroendocrine tumors.7 Recently, CgA has been shown to increase with the severity of HF8 and to be an independent predictor of mortality in patients with acute coronary syndrome.9–11 Moreover, increased CgA production has been demonstrated in cardiomyocytes of the failing myocardium.12 However, a confounding factor possibly reducing CgA’s merit as a cardiac biomarker is the increase in CgA levels seen with the use of proton pump inhibitors (PPIs).13 In contrast, CgB

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levels appear to be unaffected by PPI use and may provide additional diagnostic information to CgA in patients with neuroendocrine tumors. Additionally, colocalization of CgA and CgB in atrial tissue has been documented, linking CgB to the myocardium and possibly also cardiovascular pathophysiology. Accordingly, we hypothesized that CgB levels in the myocardium and the circulation are increased and closely regulated during HF development.

Methods

Mouse Model of HF

A permanent ligation of the left main coronary artery were performed in mice in the HF group, whereas sham-operated (sham) animals underwent the same procedure except ligation of the coronary artery. Echocardiographic examination was performed 1 week after the primary operation. The criteria for including animals in the HF group have previously been validated.

Patients With HF and Healthy Control Subjects

Eighty patients with echocardiographically documented impaired systolic function (left ventricular ejection fraction ≤50%) were included in the clinical part of this study: 70 patients from the Akershus University Hospital outpatient HF clinic and 10 patients hospitalized at Akershus University Hospital for worsening HF with dyspnea as the most prominent symptom. All patients were classified according to the New York Heart Association (NYHA) functional class system by a single investigator (H.R.). Patients with noncurable malignancy and life expectancy <1 year, acute MI, cardiac surgery, or percutaneous coronary intervention during the last 3 months were not eligible for participation in the study. Accordingly, none of the hospitalized patients were diagnosed with an acute MI.

Twenty control subjects with similar age and sex distribution as the patients were also recruited. These individuals had no history of cardiovascular disease or other concurrent disease, no current symptoms of cardiovascular disease as evaluated by 1 investigator (H.R.), and they did not use medication regularly.

The study protocol was approved by the Regional Ethics Committee before the initiation of the study. All participants gave their written informed consent prior to study commencement.

Quantitative Real-Time PCR

Gene expression was measured with premade TaqMan Gene Expression assays from Applied Biosystems (Foster City, Calif); mouse model: CgB (MM00483287_m1), CgA (MM00513431_m1), BNP (MM00435340_g1), ribosomal protein L4 (RPL4) (MM00834993_g1); and rat cardiomyocytes: CgB (Rn01514853_m1), CgA (Rn00572200_m1), BNP (Rn00580641_m1), RPL4 (Rn00820911_g1).

Immunoblotting and Immunohistochemistry

A purified polyclonal goat antihuman CgA antibody was used for immunoblotting and immunohistochemistry (SC-1409, Santa Cruz Biotechnology, Santa Cruz, Calif). This antibody showed strong and specific binding to the C-terminal region of CgB (see Online-only Data Supplement, Figure S1). A secondary antibody (diluted 1:2500) against goat (6160–05) was used for immunoblotting (Southern Biotech, Birmingham, Ala). CgA immunoreactivity in mouse myocardial sections was detected by a purified polyclonal rabbit antihuman CgA antibody (Sc-13090, Santa Cruz).

Radioimmunoassay

Plasma CgB levels were measured by an in-house made region-specific radioimmunoassay (RIA) detecting CgB439–451 plasma CgA levels by a commercial RIA identifying CgA116–439 (Euro-Diagnostica, Malmö, Sweden), and left ventricular (LV) tissue CgA levels with an in-house made region-specific RIA detecting CgA361–372.

Table 1. Descriptive Statistics of Animals

<table>
<thead>
<tr>
<th>Animal weight, day 0, g</th>
<th>HF (n=35)</th>
<th>Sham (n=29)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung weight/tibial length, g/mm</td>
<td>0.16±0.007</td>
<td>0.07±0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LV mass/tibial length, g/mm</td>
<td>0.05±0.001</td>
<td>0.04±0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RV mass/tibial length, g/mm</td>
<td>0.01±0.001</td>
<td>0.01±0.001</td>
<td>0.004</td>
</tr>
<tr>
<td>LV BNP mRNA levels</td>
<td>5.8±0.7</td>
<td>1.0±0.1</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

RV indicates right ventricle. BNP mRNA levels were analyzed in a subset of animals and are presented as mean change in the HF group (n=9) versus the sham group (n=8) (±SEM).

Cell Culture Experiments

After 24-hour starvation of the cells, neonatal rat cardiomyocytes were stimulated as previously reported for 24 hours with the following agents: forskolin (FSK) [10 μmol/L], norepinephrine (NE) [100 μmol/L], endothelin-1 (ET-1) [250 ng/mL], angiotensin II (AngII) [1 μmol/L] (all Sigma-Aldrich, St Louis, Mo), tumor necrosis factor-α (TNF-α) [10 ng/mL] (BioSource International, Camarillo, Calif), and transforming growth factor-β (TGF-β) [10 ng/mL] (R&D Systems, Minneapolis, Minn).

Statistical Analysis

Continuous data are presented as mean (±SEM) or median and interquartile range (IQR) and categorical values as counts (percent-age). Continuous variables were compared by the Student t test except circulating biomarker values that were compared by the Mann–Whitney U test due to a right-skewed distribution. Categorical variables were compared by the χ² test or Fisher exact test as appropriate. Correlations and trends were calculated using Spearman rank correlation. Probability values <0.05 were considered significant for all analyses. Statistical analyses were performed with SPSS for Windows version 14.0 (SPSS, Chicago, Ill).

Details regarding methods can be found in the online-only Data Supplement. The authors (H.R., T.O., and G.C.) had full access to the data and take responsibility for its integrity. All authors have read and agreed to the manuscript as written.

Results

Characteristics of Animals

We used a post-MI mouse HF model to study CgB production in various tissues during HF development. As shown in Table 1, the animals included in the post-MI group had clear evidence of HF and compensatory myocardial hypertrophy.

LV CgB Gene Expression Is Increased and Closely Regulated in HF

To investigate regulation of CgB gene expression in the LV during HF development, CgB mRNA levels in noninfarcted tissue of HF animals were compared with levels in sham animals. CgB mRNA levels were 5.2 times upregulated in HF animals (4.8-fold increase, P=0.025), but there was no significant correlation between LV CgA and CgB mRNA levels in HF (r=0.20, P=0.61) or sham animals (r=0.57, P=0.14).
We found no change in gene expression between nonoperated control animals and sham animals, and RPL4 gene expression was similar in the different groups.

CgB Protein Levels Are Increased in Both Noninfarcted and Infarcted LV Tissue in HF
To further study CgB production in HF, we measured CgB protein levels in the LV of HF and sham animals. Supporting our gene expression data, CgB protein levels were clearly increased in both noninfarcted and infarcted LV tissue of HF animals (110% and 70% increase, respectively, Figure 2). CgA protein levels were also increased in noninfarcted LV tissue (40% increase, \( P<0.002 \)), whereas there was no change in infarcted LV tissue.

CgB levels were not different in sham animals compared with nonoperated control animals.

CgB Levels in Non-LV Tissue Were Unaffected by HF Development
To investigate if increased CgB production is restricted to the LV during HF development, we also assessed CgB production in other tissues. No elevation of CgB levels were found in the right ventricle, pulmonary tissue, liver, spleen, kidney, stomach, colon, or skeletal muscle of HF animals (Figure 3), supporting the notion that increased CgB production may be specific to the LV in HF.

CgB Production in the Myocardium Is Confined to Cardiomyocytes
Immunohistochemistry was performed to identify the cells in the myocardium that synthesize CgB. As shown in Figure 4, fairly strong immunostaining for CgB was found in cardiomyocytes in noninfarcted myocardial tissue, whereas weaker staining was observed in the infarcted region of the LV confined to scattered surviving cardiomyocytes. No other cell type was found to produce CgB in the myocardium. Myocardial CgA production was also confined to the cardiomyocytes (Figure 4), reflecting that the chromogranins are a family of
proteins produced by the cardiomyocytes during HF development.

No or very weak staining was seen in the negative control sections stained with omission of the primary antibody or with the use of nonimmune antiserum, respectively.

CgB Gene Expression in Cardiomyocytes Is Increased by NE, AngII, and TGF-

To elaborate on the mechanism for increased cardiomyocyte CgB production in HF, we investigated the influence of signaling molecules known to be regulated in HF on cardiomyocyte CgB gene expression. NE, AngII, and TGF-

all potently upregulated cardiomyocyte CgB gene expression (Figure 5). In contrast, ET-1 and TNF-

had no effect on cardiomyocyte CgB gene expression. FSK, known to upregulate CgB gene expression in other cell types, also increased CgB gene expression in cardiomyocytes.

In a second set of experiments, these hormones and cytokines had no effect on cardiomyocyte CgA production, whereas BNP mRNA levels were increased by NE (200%), ET-1 (90%), and FSK (60%) (P < 0.001 for all). RPL4 gene expression was not affected by any of the stimuli.

Circulating Levels of CgB Are Increased and Regulated in HF

Because circulating levels of CgA have been found to predict mortality in patients with both HF and acute coronary syndrome, we next investigated if also circulating CgB levels are regulated during HF development. In the experimental model, circulating CgB levels were clearly increased in HF animals compared to sham-operated animals (median, 1.23 [IQR: 1.03 to 1.93] versus 0.98 [0.90 to 1.04] nmol/L; P = 0.003; Figure 6A), confirming that circulating levels of CgB are increased in HF. There was no difference in circulating CgB levels in nonoperated control animals and sham-operated animals.

To investigate whether CgB may have potential as a cardiac biomarker, we sought to validate our experimental data in the clinical condition. Circulating CgB levels were measured in a heterogeneous group of HF patients and compared to levels in age- and sex-matched healthy control subjects. Characteristics of patients and control subjects are presented in Table 2. In general, patients had chronic HF of mainly moderate severity with 18 months median duration of

Figure 3. CgB protein levels in non-LV tissue 1 week after MI. CgB levels in the right ventricle (A), pulmonary tissue (B), liver (C), spleen (D), kidney (E), stomach (F), colon (G), and skeletal muscle (H) were also measured by immunoblotting and presented as change in the HF group (n = 6) versus the sham group (n = 6) (± SEM). A representative blot is shown for each organ.
symptoms, a mean LV ejection fraction of 33%, a median BNP level of 223 pg/mL, and 67% of patients were classified in NYHA functional class II. Patients were treated according to updated guidelines. Median duration of HF symptoms was similar in patients recruited from the ambulatory HF clinic and hospitalized patients as the latter group consisted mainly of patients with acute-on-chronic HF (see online-only Data Supplement for further descriptive statistics of HF patients). In this cohort of patients with HF of mainly moderate severity, circulating CgB levels were significantly increased compared with the healthy control subjects (median, 1.66 [IQR: 1.48 to 1.85] versus 1.47 [1.39 to 1.58] nmol/L; \( P = 0.007 \); Figure 6B). Furthermore, circulating CgB levels increased in proportion to severity of HF as evaluated by NYHA functional class (\( P = 0.03 \) for trend). We found no difference in CgB levels in patients with HF caused by ischemic etiology compared with patients diagnosed with dilated cardiomyopathy (1.68 [1.50 to 1.80] versus 1.66 [1.47 to 1.88] nmol/L; \( P = 0.89 \)) nor between male and female HF patients (1.66 [1.49 to 1.86] versus 1.67 [1.45 to 1.85]; \( P = 0.89 \)). HF patients using PPIs had markedly increased CgA levels compared with nonusers (8.7 [5.1 to 25.0] versus 5.1 [3.6 to 8.1] nmol/L; \( P = 0.007 \)), whereas PPI use did not influence circulating CgB levels (1.62 [1.55 to 1.72] versus 1.67 [1.45 to 1.87] nmol/L; \( P = 0.98 \)). Notably, PPI users (n=11) and non-PPI users (n=69) had HF of similar severity. Circulating levels of CgB in HF patients were modestly correlated with CgA (non-PPI users: \( r = 0.31, P = 0.009 \)) and BNP levels (\( r = 0.27, P = 0.014 \)), indicating that CgB is regulated differently from other granin proteins and BNP during HF development.

Figure 4. CgB and CgA production in the myocardium. Representative photomicrographs of myocardial tissue sections of a HF mouse demonstrating CgB immunoreactivities (brown staining) detected in nonischemic cardiomyocytes bordering the infarcted zone (border zone, lower left). Similar immunostaining was also found in the remote noninfarcted myocardium (upper left). In the infarcted region (upper middle), only weak CgB immunostaining was detected. Bottom picture in the middle demonstrates very weak staining after use of nonimmune rabbit serum as control (ctr). CgA immunoreactivities (brown staining) were also confined to cardiomyocytes as illustrated by photomicrographs of the border zone and the infarcted myocardium (far right). Magnification \( \times 200 \).

Figure 5. Influence of signaling molecules known to be regulated in HF on cardiomyocyte CgB gene expression. CgB mRNA levels were measured by quantitative real-time PCR after stimulating neonatal rat cardiomyocytes for 24 hours with either PBS (n=11), FSK (n=5), NE (n=5), AngII (n=3), ET-1 (n=5), TGF-\( \beta \) (n=6), or TNF-\( \alpha \) (n=9). CgB mRNA levels are presented as means\( \pm \)SEM versus PBS-stimulated cells (ctr). *\( P < 0.01 \), **\( P < 0.001 \).
Discussion

The present study demonstrates for the first time that production of CgB, a member of the granin protein family, is regulated in accordance with disease severity in both experimental and clinical HF. Mice with post-MI HF had markedly increased LV CgB gene expression, proportional to the degree of pulmonary congestion, and CgB protein levels were also elevated in the LV during HF development. Notably, CgB production was not increased in other tissues investigated. The production of CgB in the myocardium was confined to the cardiomyocytes. Moreover, in vitro experiments revealed that NE, AngII, and TGF-β all increased cardiomyocyte CgB gene expression. Finally, we found increased circulating levels of CgB in both experimental and clinical HF.

Our criteria for including animals in the HF group have been validated previously,17 and animals in this group had clear evidence of HF as reflected by severe pulmonary congestion, significantly increased LV and right ventricular mass, and upregulated LV BNP gene expression. Interestingly, HF animals also had clearly increased LV CgB gene expression and protein levels, suggesting de novo LV CgB production in HF. Moreover, as LV CgB gene expression in HF animals was closely correlated with animal lung weights, CgB production in the myocardium appears to be regulated during HF development. Our findings are in line with another report published during progression of our work demonstrating increased LV CgB production in a mouse model of AngII-induced LV hypertrophy.22

Table 2. Descriptive Statistics of HF Patients and Control Subjects

<table>
<thead>
<tr>
<th></th>
<th>HF Patients (n=80)</th>
<th>Control Subjects (n=20)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sex, n (%)</td>
<td>64 (80)</td>
<td>16 (80)</td>
<td>1.00</td>
</tr>
<tr>
<td>Age, y, mean±SEM</td>
<td>64.1±1.4</td>
<td>60.6±1.1</td>
<td>0.24</td>
</tr>
<tr>
<td>NYHA class, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>54 (67)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>18 (23)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>8 (10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Etiology for HF, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ischemic</td>
<td>48 (60)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dilated cardiomyopathy</td>
<td>27 (34)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>5 (6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration of HF, mo, median (IQR)</td>
<td>18 (7–36)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVEF, %, mean±SEM</td>
<td>33±1</td>
<td></td>
<td></td>
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<tr>
<td>Medication, n, %</td>
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</tr>
<tr>
<td>β-blocker</td>
<td>79 (99)</td>
<td></td>
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<tr>
<td>ACEI</td>
<td>58 (73)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ARB</td>
<td>21 (26)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACEI or ARB</td>
<td>79 (99)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diuretics</td>
<td>64 (80)</td>
<td></td>
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<tr>
<td>Statin</td>
<td>48 (60)</td>
<td></td>
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</tr>
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<td>Warfarin</td>
<td>47 (59)</td>
<td></td>
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</tr>
<tr>
<td>ASA</td>
<td>43 (54)</td>
<td></td>
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</tr>
<tr>
<td>Clonidipine</td>
<td>9 (11)</td>
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<tr>
<td>Aldosterone antagonist</td>
<td>16 (20)</td>
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<tr>
<td>Digitals</td>
<td>30 (38)</td>
<td></td>
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<tr>
<td>Amiodarone</td>
<td>10 (13)</td>
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</tr>
<tr>
<td>Nitrate</td>
<td>10 (13)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPI</td>
<td>11 (14)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRT</td>
<td>14 (18)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICD</td>
<td>14 (18)</td>
<td></td>
<td></td>
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<tr>
<td>CgA levels, nmol/L, median (IQR)</td>
<td>5.5 (3.6–8.4)</td>
<td>4.5 (4.0–5.3)</td>
<td>0.11</td>
</tr>
<tr>
<td>BNP levels, pg/mL, median (IQR)</td>
<td>223 (119–617)</td>
<td>26 (13–37)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

IQR indicates interquartile range; LVEF, LV ejection fraction; ACEI, ACE inhibitor; ASA, acetyl salicylic acid; CRT, cardiac resynchronization therapy; and ICD, implantable cardioverter-defibrillator.
we demonstrated that CgB production is confined to the cardiomyocytes in the myocardium. We postulate that the myocardium may be an important source of increase in circulating CgB levels found in HF because (1) CgB production was increased in a closely regulated manner in the LV during HF development, (2) no regulation of CgB production was found in any other organ investigated, and (3) CgB and the chromogranins are considered marker-proteins for exocytosis in numerous cell types.23

The mechanism for increased CgB production in cardiomyocytes was investigated in vitro. We used FSK as a positive control as the CgB promoter contains a cAMP response element.24 NE has been found to activate cAMP response element transcription sites through both α1- and β-adrenergic receptor signaling,25,26 and NE-induced increase in CgB gene expression may reflect CgB stimulus-transcription-secretion coupling in the myocardium during HF, a mechanism known to be important for CgB production and secretion in nonmyocardial tissue.21 Because circulating NE levels in HF are increased in proportion to patient NYHA functional class,27 the effect of NE on cardiomyocyte CgB production, and possibly also secretion, may explain the close association found between CgB levels in the myocardium and plasma and indices of severity of HF. However, additional studies are needed to precisely characterize the effect of NE on cardiomyocyte CgB exocytosis and the relative importance of NE versus the effect by AngII and TGF-β on CgB production and secretion.

To our knowledge, no previous study has examined CgB production or circulating CgB levels during HF development. Increased CgB production has been found in neuroendocrine tumors,28 and circulating CgB levels have been proposed as a potential biomarker in patients with neuroendocrine tumors.14,29 The demonstration of increased circulating CgB levels during HF development suggests that CgB may potentially represent a novel cardiac biomarker. This notion is supported by regulation of circulating CgB levels in proportion to NYHA functional class. However, the relative increase in circulating CgB levels in HF individuals was larger in the animal model than in the clinical study, indicating that CgB production may be more potently induced in the early phase of HF development compared with the stable, chronic phase. A trend for higher CgB levels in hospitalized than in ambulatory patients (Supplemental Table S1 and Figure S2) is also compatible with the theory that acute decompensation may lead to higher CgB levels than observed in the chronic state. Furthermore, contemporary HF therapy may have contributed to lower CgB levels in stable ambulatory patients.

CgB has been reported to be robust and stable in vitro, with only 3.7% degradation found in plasma samples stored at room temperature for 24 hours.29 In addition, circulating CgB levels were unaffected by PPI use, a medication currently recommended as a prophylactic measure in many patients with heart disease,30 and with well-known stimulatory effects on CgA production.13 Moreover, few other conditions are known to be associated with increased circulating CgB levels. In fact, except for the rare neuroendocrine tumors,14,29,31-32 confounding by other diseases does not seem to represent a major problem for use of CgB as a marker of cardiac status.

Even in prostate cancer patients with metastatic disease and tumors with widespread neuroendocrine differentiation, only 14% of patients had increased circulating CgB levels, whereas 59% of patients had increased CgA levels.33 Taken together, this information suggests that the collection and handling of blood samples is relatively simple for CgB.

Reports on circulating CgB levels in association with cardiovascular disease are scarce, but 1 study investigated the association between presurgery CgB levels and postsurgery atrial fibrillation in patients undergoing coronary by-pass grafting.34 The authors found no difference in CgB levels between patients developing arrhythmias and patients in stable sinus rhythm after surgery, opposing the hypothesis that CgB levels could reflect autonomous imbalance. In contrast, a polymorphism in the CgB gene was reported to be the strongest predictor of enhanced vasoconstriction in a study assessing association between single nucleotide polymorphisms in the adrenergic pathway and venous vasoconstriction.35 The net effect of increased circulating CgB levels in HF patients with regard to autonomic nervous system tone is not clear and will require additional investigations. Increased CgB levels may also have direct effects on the cardiomyocytes and the myocardium. CgB, in contrast to CgA, translocates to the nucleus and has been found to regulate gene transcription, including increasing myocyte enhancer factor 2 (MEF2) production,36 a well-known prohypertrophic factor in the myocardium.37 CgB is also considered an important modulator of the inositol 1,4,5-trisphosphate receptor (IP3R),38 and recently, CgB was shown to regulate AngII-induced myocardial hypertrophy in vitro by modifying Ca2⁺ release from the sarcoplasmatic reticulum through the IP3R.22 The augmentation of IP3R-mediated Ca2⁺ release by CgB may also be of importance for the generation of ventricular arrhythmias in HF because the IP3R recently was shown to regulate ryanoide receptor stability during myocardial remodeling.39 Because both CgB and the IP3R are upregulated in the failing myocardium, this CgB-IP3R complex may be responsible for altered Ca2⁺ homeostasis resulting in increased susceptibility to arrhythmias. Additionally, as CgB production in cardiomyocytes is closely regulated by NE, CgB levels in the myocardium, and potentially also in the circulation, may integrate information on cardiomyocyte NE stimulation and IP3R activity, both risk factors for development of ventricular arrhythmias. Still, similar to the situation of other recently proposed markers, more work is needed to establish CgB as a clinically useful HF biomarker (Table 3). Of note, CgA also binds to the IP3R but seems to be inferior to CgB for regulation of IP3R activity.38 In contrast, fragments of CgA have been found to affect several important processes in HF such as myocardial contractility, endothelial function, and arterial tone,41 whereas such effects have not been recognized for CgB fragments. A comparison of CgA and CgB is provided in Table 4; however, the relative merit of circulating CgA and CgB as cardiac biomarkers must be further characterized.

The main limitation of this study is lack of data relating CgB to outcome in patients with HF. The HF patient cohort is of modest size and consists of patients with both ischemic and nonischemic HF, and acute and chronic HF. Our data for
CgB as a cardiac biomarker clearly needs to be validated in larger HF cohorts, both regarding diagnostic and prognostic utility. Consequently, the current study should be considered a phase one/proof of concept investigation of CgB as a cardiac biomarker.42

In conclusion, we have found increased CgB production in LV cardiomyocytes from post-MI HF animals proportional to HF severity. Because cardiomyocyte CgB gene expression was increased by NE, AngII, and TGF-β, myocardial CgB levels may reflect alterations in important hormonal and paracrine mediators in HF. Furthermore, circulating CgB levels were increased in both HF animals and in patients with HF of mainly moderate severity, again proportionate to HF severity. Moreover, as CgB production was not altered in other organs investigated, the increased circulating levels may at least partly reflect enhanced CgB production in the LV. Finally, because circulating CgB levels were not affected by PPI use and increased in HF patients, CgB may represent a potential new HF biomarker, but this must be validated in larger clinical studies.

Acknowledgments
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Disclosures
Drs Røsjø, Stridsberg, Omland, and Christensen are partners in a patent application filed by the University of Oslo regarding the use of CgB as a cardiac biomarker. Dr Stridsberg has a contract with Euro-Diagnostica AB (Malmö, Sweden) for developing a commercially available method for measuring CgB.

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47. Sekiya K, Ghati ME, Alahahdun MI, Bishop AE, Hamid QA, Ibayashi

**CLINICAL PERSPECTIVE**

The cardiac biomarkers used routinely in clinical practice today are proteins specific to the diseased myocardium, either released during cell necrosis (cardiac-specific troponins) or secreted secondary to cardiomyocyte strain (B-type natriuretic peptide). However, there is still a need for markers reflecting other pathophysiological processes in heart disease. We have identified chromogranin B (CgB) as a protein that is closely regulated in the left ventricle in heart failure (HF), while production was unaltered in other tissues investigated. Circulating levels of CgB were also elevated in patients with HF compared with healthy control subjects, and levels increased in proportion to severity of HF as evaluated by NYHA functional class. Plasma CgB levels correlated only modestly with B-type natriuretic peptide and chromogranin A (CgA) levels, another granin protein and novel HF biomarker, indicating that CgB is regulated by other mechanism than B-type natriuretic peptide and CgA in HF. As CgB is closely associated with Ca²⁺ signaling, CgB could potentially be a marker providing information on processes currently not reflected by established HF biomarkers. However, the role of CgB in HF development and as a cardiac biomarker still requires additional experimental and clinical studies before clinical utility may be established.
Chromogranin B in Heart Failure: A Putative Cardiac Biomarker Expressed in the Failing Myocardium

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Chromogranin B in Heart Failure: a Putative Cardiac Biomarker Expressed in the Failing Myocardium

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S2
Supplemental Methods

Mouse Model of HF

Six week old C56BL/6 mice (Taconic, Skensved, Denmark) were anesthetized with 0.2 mg propofol in the tail vein before being trachetomized, connected to an animal ventilator, and ventilated with a mixture of 98% oxygen and 2% isoflurane. Via a left-sided thoracotomy, pericardectomy was performed followed by ligation of the left main coronary artery in the HF group. Sham-operated (sham) animals underwent the same procedure except ligation of the coronary artery. One week after the primary operation, a full echocardiographic examination was performed while animals were anesthetized breathing a gas mixture of oxygen and isoflurane supplied via a facemask. Criteria used for including animals in the HF group were: 1) MI >40 % of the circumference of the LV, 2) left atrial diameter >2.0 mm, 3) >35 % increase in lung weight compared to the sham group (e.g. lung weight >0.2 g).

After sacrificing the animals one week post-surgery, hearts were removed, blotted dry and dissected into the right and LV. The LV was further divided into the infarcted and non-infarcted part in HF animals. Lung tissue and tissue samples from liver, spleen, kidney, stomach, part of the colon and the anterior tibial muscle were also collected. Tissue intended for qRT-PCR or immunoblotting were immediately frozen in liquid nitrogen and stored at 70°C until use, while hearts collected for immunohistological analysis were fixed overnight in 4% formalin, washed in 30% ethanol and stored in 70% ethanol at 4°C before use.

To evaluate the effect on gene expression and protein levels by anesthesia and surgery per se sham animals were compared to age-matched non-operated animals for all experiments.
The study was performed according to the recommendations given by the European Council for Laboratory Animal Science and approved by the Norwegian Council for Animal Research.

**HF Patients**

All patients were included at a single center, Akershus University Hospital, a secondary referral and teaching hospital in metropolitan Oslo, Norway, with a catchment area of approximately 320,000 people. The seventy patients recruited from the ambulatory HF clinic were at the time of study inclusion managed solely by scheduled 30 minutes visits to specially trained nurses in the outpatient clinic. Of these seventy patients, twelve had been hospitalized for worsening HF during the last three months. We also included ten patients with a previous diagnosis of HF that were hospitalized at the time of patient recruitment: for all of these ten patients the index hospitalization was HF related as classified by the treating physician (International Classifications of Diseases, 10th revision, World Health Organization: code I50.1 or I50.9).

Troponin T (TnT) values were measured in all hospitalized, but not in ambulatory patients. No patient had evidence of acute myocardial infarction as defined by the recent universal definition of myocardial infarction: Rise and/or fall of TnT values exceeding the 99th percentile of the upper reference limit.¹

A transthoracic echocardiogram had been obtained within the last 18 months in all but 3 patients (4%) who had been clinically stable during this period, i.e. no change in severity of HF symptoms or NYHA functional class during the last 12 months and no hospitalization for worsening HF during the last 24 months. One of these patients had been evaluated by myocardial single photon emission computed tomography (SPECT) during the last 12 months.
and had a LVEF of 20% and no evidence of reversible myocardial ischemia. Patients with HF and preserved LV function (>50%) were not included in the clinical cohort. Seventy-three patients (91%) had undergone coronary angiography for diagnostic purposes.

**Blood Samples**

Blood samples from humans were drawn from an antecubital vein, while blood samples from animals were collected from the vena cava inferior after a laparotomy of anesthetized animals breathing a combination of oxygen and isoflurane. Blood samples were immediately put on ice, centrifuged within 30 minutes, and the plasma stored at −70°C. Plasma B-type natriuretic peptide (BNP) levels were measured by a two-step sandwich immunoassay (Architect® BNP assay, Abbott Diagnostics, Abbott Park, IL).

**qRT-PCR**

Tissue (20-35 mg) from the non-infarcted region of the LV from 9 HF mice and 8 sham mice was used for RNA extraction. Homogenization was performed with the Mixer Mill MM 300 system (RETSCH, Haan, Germany) after adding 175 μl lyses buffer and a 5 mm stainless steel bead (Qiagen, Hilden, Germany) to the samples. Total RNA was extracted by the use of the SV Total RNA Isolation System (Promega Corporation, Madison, WI). RNA concentration was measured with the NanoDrop system (NanoDrop Technologies, Wilmington, DE) and RNA quality evaluated with the Agilent BioAnalyzer 2100 (Agilent Technologies Inc., Santa Clara, CA). cDNA was produced from 5 μg RNA with the High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA); the GeneAmp PCR system 9700 thermal cycler (Applied Biosystems) was used for the reverse transcription. The results were detected on a 7900 HT Real-Time PCR System (Applied Biosystems), and the relative gene expression was determined by using the standard curve method.² The standard
curve for CgB was plotted from neonatal mouse brain tissue, while myocardial tissue was used for plotting BNP and RPL4 standard curves. All samples were run in triplicate and RPL4 served as an internal control. Levels are presented as change from the mean in the sham group (fold change).

1-D Gel Electrophoresis and Immunoblotting

Frozen myocardial tissue samples were homogenized in a cold lysis buffer containing 210 mM sucrose, 40 mM NaCl, 30 mM Hepes, 5 mM EDTA, 1% Tween-20, and different protease inhibitors (Complete EDTA-free protease inhibitor cocktail, Roche Diagnostics, Basel, Switzerland). Mechanical homogenization was performed with the Mixer Mill MM 300 system with insoluble material removed after centrifugation at 14000 x g. The lysates were added 1% SDS as a final concentration. Total protein content was measured with the micro BCA protein assay kit (Pierce Biotechnology, Rockford, IL) according to the manufactures protocol. Prior to gel loading, lysates were denatured for 5 minutes at 100°C after mixing 20-30 ug protein with SDS gel-loading buffer (50% sucrose, 7.5% SDS, 0.0625M Tris-HCl, pH 6.8, 2mM EDTA, pH 7.5, 3.1% DTT, and 0.01% bromophenolblue). After protein loading, polyacrylamide gels (10-12 %) were run approximately for 75 minutes at 200 V, however, with some variation as electrophoresis length was chosen according to the protein of interest. Precision Plus Protein Dual Color Standard (161-0374) was used as the molecular marker (Bio-Rad Laboratories Inc., Hercules, CA) and included in all gels. Proteins separated on gels were transferred to Hybond-P PVDF membranes (RPN303F, Amersham Biosciences Europe, Freiburg, Germany) using a Mini Trans-Blot Cell system (Bio-Rad Laboratories Inc.), incubated at room temperature (RT) with 5% skimmed dry milk diluted in Tris-buffered saline containing 0.1% Tween (TBS-t) for 2 hours to avoid unspecific antibody binding. Primary antibodies were diluted in 5% skimmed dry milk in TBS-t before incubation.
overnight at 4°C. The next day secondary antibodies diluted in 5% skimmed dry milk in TBS-t were incubated to the membranes for 1 hour at RT. Membranes were washed three times for 5-15 minutes in TBS-t in between and after all incubation steps. The roller mixer (444-1607, VWR International) was used for incubations.

For visualizing immunostained proteins the ECL Plus Western Detection System (Amersham Biosciences Europe, Freiburg, Germany) and an ImageReader LAS 3000-mini digital detector (Fujifilm, Tokyo, Japan) were used with densitometry of immunostained bands measured with MultiGauge (Fujifilm). Equal protein loading on gels was controlled by using anti-glyceraldehyde-3-dehydrogenase (GAPDH) as an internal control (2118, Cell Signaling Technology, Beverly, MA). We used a secondary anti-goat antibody for GADPH analysis (4030-05, Southern Biotech, Birmingham, AL).

In general, protein lysates from HF and sham animals were loaded in every second well in the acrylamide gels to avoid differences due to technical difficulties, six individuals per group were maximum per gel. For myocardial tissue with n>6 in HF and sham groups; blots were compared by normalizing bands against three individuals whom had samples run on all gels. CgB levels are presented as change from the mean in the sham group. As CgB has a highly acidic charge, CgB migrates slower in the SDS-PAGE system than predicted from its calculated molecular weight. By immunoblotting, strong protein bands were found for the positive controls with molecular weights corresponding to what has earlier been reported as the full length CgB molecule in the SDS-PAGE system (100-120 kDa). Total protein extracts from neonatal mouse brain or a rat pheochromocytoma cell line (PC12 cell line, sc-2250, Santa Cruz Biotechnology, Santa Cruz, CA) were included in the gels as positive controls.
Radioimmunoassay on Tissue Homogenate

Frozen LV tissue samples were homogenized as described in the previous section. CgA protein levels were measured with an in-house made region-specific RIA detecting CgA361-372 from 50 µg of total protein homogenate. All samples were measured in duplicate and no sample had CgA levels below the detection limit (<2 fmol/tube). LV tissue samples were homogenized as described in the previous section.

Immunohistochemistry

The CgB (sc-1489, Santa Cruz) and CgA (Sc-13090, Santa Cruz) antibodies were followed by anti-goat IgG (Vector Laboratories, Burlingame, CA). The CgA antibody was a purified polyclonal rabbit anti-human CgA raised against the C-terminal part of CgA. The immunoreactivities were further amplified using avidin-biotin-peroxidase complexes (Vectastain Elite kit, Vector Laboratories). Diaminobenzidine was used as the chromogen in a commercial metal enhanced system (Pierce Biotechnology). The sections were counterstained with hematoxylin. Omission of the primary antibody or use of non-immune rabbit serum served as negative controls.

Cell Culture Experiments

Neonatal rat cardiomyocytes were isolated from neonatal (1-3 days) Wistar rats (Taconic). Mechanical disruption of cells and evaluation of RNA concentrations and quality were performed as described under section qRT-PCR, except that total RNA was isolated from the neonatal rat cardiomyocytes by the use of the RNeasy mini kit (Qiagen, Valencia, CA). Reverse transcription reactions were performed with iScript Select cDNA Synthesis Kit (Bio-Rad Laboratories Inc.).
Gene expression in stimulated cells is presented as change vs. gene expression in PBS stimulated cells (fold change). Samples were run in duplicate or triplicate and RPL4 gene expression was used as an internal control.

**Mapping of Binding Site for CgB Antibody Used in Immunoblotting and Immunohistochemistry**

**Peptide Synthesis**
Mouse CgB (NP_031720) was synthesized as 20-mer peptides with five amino acids offsets on cellulose membranes using a Multipep automated peptide synthesizer (INTAVIS Bioanalytical Instruments AG, Cologne, Germany) as described.  

**Immunoblot Analysis**
The membranes were blocked in 5% non-fat dry milk for 60 minutes at room temperature, incubated overnight at 4°C with primary antibody in TBS-t, washed five times 5 minutes in TBS-t and incubated with a horseradish-peroxidase-conjugated secondary antibody in 5% non-fat dry milk for 60 minutes at room temperature. Blots were developed by using ECL Plus (GE HealthCare, RPN2132). The chemiluminescence signals were detected by Las-4000 (Fujifilm).

**Antibodies**
Immunoblotting was carried out using goat anti-CgB (1:100 dilution, sc-1489, Santa Cruz). Donkey anti-goat IgG HRP affinity purified polyclonal antibody (1:2500 dilution, HAF109, R&D Systems, Minneapolis, MN) was used as secondary antibody.
Evaluation of CgB Antibody Used for Immunoblotting and Immunohistochemistry by Blocking Peptide

1-D gel electrophoresis and immunoblotting were performed as earlier described except that a blocking peptide (sc-1489 P, Santa Cruz) specific for our CgB antibody was preadded in excess to the primary antibody solution aimed to one half of the Hybond-P PVDF membrane prior to transferring proteins from the gel.

Supplemental Results

Characteristics of Patients Included From the Ambulatory HF Clinic and the Hospitalized HF Patients

The hospitalized HF patients included in the study were older and had more severe HF as evaluated by LVEF, BNP levels, and NYHA functional class compared to the other HF patients (Table S1). A higher proportion of these patients were also treated with cardiac resynchronization therapy, digitalis, and ACE-inhibitors (ACEI). However, there were no difference in combined ACEI/AngII receptor blocker medication between patients admitted from the ambulatory HF clinic and the hospitalized HF patients (Table S1). Diuretic and nitrate use, and circulating CgA levels were of borderline difference. There was also a trend towards higher levels of circulating CgB in the hospitalized HF patients compared to the other HF patients, but the difference did not reach statistical significance (median 1.72 [interquartile range: 1.54-1.93] vs. 1.65 [1.46-1.82] nmol/L, p=0.27, Table S1 and Figure S2).

Epitope Mapping of Anti-CgB Antibody (sc-1489)

Strong and specific binding was observed to the amino acid sequence EEKKELENLAAMDLELQKIAEKFSQRG (Figure S1, lower panel) where underlined amino acids indicate the core epitope. The identified residues are located to the extreme C-terminus.
in CgB and are consistent with the source information from the manufacturer (Santa Cruz). Immunoblotting without incubation with primary antibody was used as a negative control (Figure S1, upper panel).

**Evaluation of CgB Antibody by Blocking Peptide**

No bands were detected on the part of the membrane pretreated with the blocking peptide (Figure S3), indicating that our CgB antibody is sensitive and specific for CgB. Strong bands corresponding to what has earlier been reported as the full length CgB molecule in the SDS-PAGE system (100-120 kDa) were found on the non-pretreated half of the membrane.
**Supplemental Table S1. Descriptive statistics of patients included from the ambulatory HF clinic and hospitalized HF patients**

<table>
<thead>
<tr>
<th></th>
<th>Ambulatory HF clinic (n=70)</th>
<th>Hospitalized HF patients (n=10)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sex (no, %)</td>
<td>57 (81%)</td>
<td>7 (70%)</td>
<td>0.40</td>
</tr>
<tr>
<td>Age (years, mean ± SEM)</td>
<td>62.7 ± 1.5</td>
<td>73.5 ± 2.4</td>
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<tr>
<td>NYHA class (no, %)</td>
<td></td>
<td></td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>II</td>
<td>54 (77%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>15 (22%)</td>
<td>3 (30%)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>1 (1%)</td>
<td>7 (70%)</td>
<td></td>
</tr>
<tr>
<td>Etiology for HF (no, %)</td>
<td></td>
<td></td>
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<tr>
<td>Ischemic</td>
<td>39 (56%)</td>
<td>8 (80%)</td>
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<tr>
<td>Dilated cardiomyopathy</td>
<td>25 (36%)</td>
<td>2 (20%)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>6 (8%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>Duration of HF (months, median, IQR)</td>
<td>18 (11-36)</td>
<td>18 (6-62)</td>
<td>0.76</td>
</tr>
<tr>
<td>LVEF, % (mean ± SEM)</td>
<td>34 ± 1</td>
<td>26 ± 3</td>
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<tr>
<td>Medication (no, %)</td>
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<tr>
<td>β-blocker</td>
<td>69 (99%)</td>
<td>10 (100%)</td>
<td>0.70</td>
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<tr>
<td>ACEI</td>
<td>48 (69%)</td>
<td>10 (100%)</td>
<td>0.04</td>
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<tr>
<td>ARB</td>
<td>21 (30%)</td>
<td>0 (0%)</td>
<td>0.04</td>
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<tr>
<td>ACEI or ARB</td>
<td>69 (99%)</td>
<td>10 (100%)</td>
<td>0.70</td>
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<tr>
<td>Diuretics</td>
<td>54 (77%)</td>
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<td>Statin</td>
<td>44 (63%)</td>
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<td>No (N%)</td>
<td>P</td>
</tr>
<tr>
<td>--------------------------</td>
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<tr>
<td>ASA</td>
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<td>Amiodarone</td>
<td>8 (11%)</td>
<td>2 (20%)</td>
<td>0.44</td>
</tr>
<tr>
<td>Nitrate</td>
<td>7 (10%)</td>
<td>3 (30%)</td>
<td>0.07</td>
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<tr>
<td>PPI</td>
<td>9 (13%)</td>
<td>2 (20%)</td>
<td>0.54</td>
</tr>
<tr>
<td>CRT</td>
<td>10 (14%)</td>
<td>4 (40%)</td>
<td>0.05</td>
</tr>
<tr>
<td>ICD</td>
<td>12 (17%)</td>
<td>2 (20%)</td>
<td>0.82</td>
</tr>
<tr>
<td>CgB levels, nmol/L (med, IQR)</td>
<td>1.65 (1.46-1.82)</td>
<td>1.72 (1.54-1.93)</td>
<td>0.27</td>
</tr>
<tr>
<td>CgA levels, nmol/L (med, IQR)</td>
<td>5.1 (3.6-8.3)</td>
<td>7.9 (6.2-12.2)</td>
<td>0.08</td>
</tr>
<tr>
<td>BNP levels, pg/mL (med, IQR)</td>
<td>210 (107-368)</td>
<td>1470 (874-2515)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

NYHA indicates New York Heart Association functional class; IQR, interquartile range; LVEF, left ventricular ejection fraction; ACEI, ACE-inhibitor; ARB, AngII receptor blocker; ASA, acetyl salicylic acid; PPI: proton pump inhibitor; CRT, cardiac resynchronization therapy; ICD, implantable cardioverter-defibrillator; CgB, chromogranin B; CgA, chromogranin A; and BNP, B-type natriuretic peptide.
Supplemental Figure S1

- anti-chromogranin B (sc-1489)

+ anti-chromogranin B (sc-1489)

EELKELENLAAMDLELQKIAEKFSQRG
Supplemental Figure S2

Ambulatory HF clinic vs. Hospitalized HF patients

CgB levels (nmol/L)
Supplemental Figure S3

<table>
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<tr>
<th>Blocking peptide +/-</th>
<th>Sham</th>
<th>HF</th>
<th>Sham</th>
<th>HF</th>
<th>Sham</th>
<th>HF</th>
<th>Sham</th>
<th>HF</th>
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</thead>
<tbody>
<tr>
<td>CgB (100 kDa)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</table>
Supplemental Figure legends

Supplemental Figure S1. Epitope mapping of anti-CgB, sc-1489. CgB residues important for antibody binding were identified by overlaying an array of immobilized CgB 20-mer peptides with anti-CgB, sc-1487 (lower panel). Strong and specific binding was observed to the amino acid sequence EEKKELENLAAMDLELQIAEKFSQRG which is located to the C-terminal end of CgB and is consistent with the source information of the manufacturer. The given amino acids are relevant for binding and underlined amino acids indicate the core epitope. Immunoblotting without incubation with primary antibody was used as a negative control (upper panel).

Supplemental Figure S2. Circulating CgB levels in patients admitted from the ambulatory HF clinic and hospitalized HF patients. The horizontal line within the box represents the median level, the boundaries of the box the 25th and 75th percentile levels, and the whiskers range (maximum value restricted to 1.5 x interquartile range from the median).

Supplemental Figure S3. CgB antibody evaluated by use of a blocking peptide. No bands were detected on the part of the membrane pretreated with the blocking peptide (left side) while strong bands corresponding to what has earlier been reported as the full length CgB molecule in the SDS-PAGE system (100 kDa) were found on the non-pretreated part (right side).
Supplemental References


(2) Yuan JS, Reed A, Chen F, Stewart CN, Jr. Statistical analysis of real-time PCR data. BMC Bioinformatics. 2006;7:85.
