Elevated Levels of the Mediator of Catabolic Bone Remodeling RANKL in the Bone Marrow Environment: Link Chronic Heart Failure with Osteoporosis

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Background—Chronic heart failure (CHF) is associated with a 4-fold increased risk for osteoporotic fractures. Therefore, we sought to identify the pathophysiological link between chronic heart failure and catabolic bone remodeling.

Methods and Results—In a total cohort of 153 subjects (123 patients with CHF, 30 patients with coronary artery disease and preserved cardiac function) as well as mice with heart failure, bone marrow (BM) plasma levels of the catabolic receptor activator of NF-κB ligand (RANKL), and its antagonist, osteoprotegerin were measured. The osteoclast inducing activity of BM plasma was tested in cell culture. BM plasma levels of RANKL and of the ratio RANKL/osteoprotegerin were significantly elevated in patients with CHF. On multivariate regression analysis, parameters of severity and duration of heart failure were independent determinants of elevated BM plasma RANKL levels. BM plasma levels of RANKL were directly correlated with the systemic marker of bone turnover C-telopeptide of type 1 collagen (r=0.6; P<0.001). Alterations in BM plasma levels of RANKL/osteoprotegerin were confirmed in a mouse model of postinfarction heart failure. Stimulation of human mesenchymal cells with BM plasma obtained from CHF patients increased the formation of osteoclasts, and this effect was blocked by the RANKL inhibition.

Conclusions—CHF is associated with a profound and selective elevation of the bone resorption stimulating RANKL within the BM microenvironment. These data for the first time disclose a direct pathophysiological pathway linking CHF with catabolic bone remodeling associated with an increased osteoporotic fracture risk.


Key Words: RANKL ▼ OPG ▼ heart failure ▼ bone marrow ▼ osteoporosis

Heart failure remains one of the major causes of mortality in industrialized countries.1,2 Recent evidence suggests that chronic heart failure is associated with factors that may contribute to accelerated bone loss and subsequent fractures. In a large population-based cohort study, chronic heart failure was associated with a 4-fold increased risk for osteoporotic fractures.3 In addition, x-ray imaging of the vertebral spine demonstrated a significantly increased rate of osteoporotic vertebral compression fractures in patients with chronic heart failure.4 Although previous studies attributed the increased fracture risk in patients with heart failure to a greater propensity to falls as well as immobilization with concomitant decrease in muscle strength and postural stability,5 a recent population-based twin study6 demonstrated that genetic predisposition is a major determinant of the excess fracture rate. In fact, most of the overall increased rate of hip fracture after heart failure appears to be explained by genes or by early environmental sharing, independent of comorbidity and lifestyle habits.6 Thus, common pathophysiological mechanisms are most likely to contribute to the association between heart failure and increased osteoporotic fracture risk.

Clinical Perspective on p 777

Osteoporosis results from an imbalance in skeletal remodeling that favors bone resorption over bone formation.3 Bone resorption is dependent on a cytokine known as receptor activator of NF-κB ligand (RANKL), a tumor necrosis factor family member that is essential for osteoclast formation, activity, and survival in normal and pathological states of bone.
remodeling. The catabolic effects of RANKL are counteracted by osteoprotegerin (OPG), which binds RANKL and, thereby, prevents activation of its single cognate receptor called RANK. Thus, the relative balance of RANKL and its physiological inhibitor OPG fine tunes bone homeostasis and remodeling.

To disclose a potential common pathophysiological mechanism linking chronic heart failure with a dysregulated balance of mediators of bone remodeling within the local environment in the bone, we measured the levels of RANKL and OPG in the bone marrow plasma.

Methods

Study Population

Patients with chronic systolic heart failure were recruited from the patient cohort included in studies assessing the effect of intracoronary bone marrow-derived mononuclear cell infusion for treatment of chronic heart failure (ClinicalTrials.gov; accession numbers: NCT 00289822, NCT 00284713, NCT 00326989, NCT 00962364). Systolic heart failure was defined as left-ventricular ejection fraction ≤45% assessed by quantitative echocardiographic or angiographic analysis. Patients with chronic postischemic heart failure had angiographically documented coronary artery disease (CAD) and persistent regional left-ventricular dysfunction. Clinical status was documented at the time of bone marrow aspiration: heart failure parameters were assessed by established standards (New York Heart Association-class), left-ventricular ejection fraction was determined by quantitative left-ventricular angiography or in case of a suspected left-ventricular thrombus (n=11) by quantitative echocardiography. N-terminal pro-Brain natriuretic peptide (NT-pro-BNP) and high-sensitive C-reactive protein (hsCRP) were measured by commercially available standard assays (NT-pro-BNP: ELECSYS2010 analyzer; F. Hoffmann-La Roche Diagnostics, Basel, Switzerland; hsCRP Roche Modular: F. Hoffmann-La Roche Diagnostics, Basel, Switzerland). All patients had to be in stable condition for at least 3 months before inclusion into the study. Patients with ischemic events within the preceding 3 months were excluded. In addition, patients with acutely decompensated heart failure with New York Heart Association functional class IV were excluded from the study.

The CAD control group consisted of patients with angiographically documented CAD, but normal left-ventricular ejection fraction and absence of any echocardiographic findings of hemodynamically relevant valvular heart disease or any evidence for diastolic heart failure. General exclusion criteria were a history of leukopenia, thrombocytopenia, or severe hepatic and renal dysfunction as well as evidence for inflammatory or malignant disease. None of the subjects participating in the study received treatment with bisphosphonates or steroids. The ethics review board of the Johann Wolfgang Goethe University of Frankfurt, Germany, approved the protocols, and the study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from each patient.

Analysis of Bone Marrow Plasma

Fifty milliliter of bone marrow were aspirated from the iliac crest under local anesthesia. Bone marrow plasma was obtained by centrifugation of heparinized bone marrow aspirates at 800 g and was kept frozen at −80°C until further use. Bone marrow plasma levels of RANKL and OPG were measured by high-sensitive enzyme-linked immunosorbent assays (RANKL: Immundiagnostik AG, Bensheim, Germany; OPG: R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany) according to the manufacturers’ recommendations. The interassay coefficients of variation were as follows: RANKL: 8.2%; OPG: 9.1%. In addition, in a subset of subjects, in whom peripheral blood samples obtained at the time of bone marrow aspiration were available, serum levels of parathyroid hormone (PTH) (n=91), RANKL (n=60), and C-telopeptide of type I collagen (CTX) (n=68) were determined in samples obtained from the peripheral blood by high-sensitive enzyme-linked immunosorbent assays (PTH: DRG Instruments GmbH, Marburg, Germany; CTX: Roche Diagnostics GmbH, Mannheim, Germany; RANKL: Immundiagnostik AG, Bensheim, Germany) after an overnight fast.

Animal Experiments

As patients with chronic heart failure have multiple comorbidities, which may confound the observed results, we performed a series of animal studies to confirm the clinical observations with respect to the mediators of bone remodeling in the bone marrow plasma without these multiple potential confounders. For this purpose, chronic heart failure was experimentally induced. BALB/cOlaHsd mice, 8 to 10 weeks old, with a body weight of 20 to 25 gram, underwent left coronary artery ligation to induce myocardial infarction, as described earlier. At week 8 after myocardial infarction, shortly before the mice were sacrificed, echocardiographic studies were performed, a time point mimicking the clinical scenario of chronic postinfarction heart failure. Echocardiographic studies were performed under light anesthesia with spontaneous respiration using isoflurane. An ultrasonographer experienced in rodent imaging and blinded to the mouse treatment performed the echocardiography, operating a Toshiba Aplo and a 15 MHz transducer. Mice with severely impaired cardiac function (fractional shortening <20%) were compared with sham-operated mice (n=5) or mice with only mildly impaired cardiac function (fractional shortening ≥20%; control group). Bone marrow plasma was collected by flushing the femurs with 500 µl phosphate buffered saline. Aspirates were centrifuged at 800 g for 10 minute, supernatants were stored at −80°C, and cytokine levels were measured by high-sensitive enzyme-linked immunosorbent assays (RANKL: Immundiagnostik AG, Bensheim, Germany; OPG: R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany) according to the manufacturers’ recommendations.

The Standing Committee on Animal Research of our institution approved the animal study protocol. The investigation conforms with the guide for the care and use of laboratory animals published by the US National Institutes of Health.

Cell Culture Study

Mesenchymal stromal cells were isolated from bone marrow mononuclear cells from healthy volunteers as previously described. Mesenchymal stromal cells were incubated with 25% bone marrow plasma (4× concentrated) with or without the RANKL-specific monoclonal antibody Denosumab (100 µg/mL Prolia, Amgen GmbH München, Germany) for 7 days. Osteoclasts were identified by Tartrate-Resistant Acid Phosphatase as previously described.

Statistical Analyses

If not stated otherwise, continuously distributed variables were reported as mean±SD, categorical variables by absolute and relative frequencies. Preliminary data analyses revealed that the bone marrow plasma levels of RANKL and OPG followed an asymptotically normal distribution. Between study group differences were tested either by a χ² test or a 2 sample t test for unequal variances. Bivariate correlations were calculated by the Pearson correlation coefficient. Univariate and multivariate linear regression analyses were conducted for investigating the association of bone marrow plasma levels and clinical characteristics. Statistically significant predictor variables (P<0.05) from univariate regression analyses served as predictor variables in the 2 multivariate models. Bone marrow plasma levels of chronically infarcted mice were compared with age-matched mice by Wilcoxon-Mann-Whitney-Test; group differences in the cell culture experiments were calculated by ANOVA after calculation of variance by Levine test. Statistical significance was assumed, if a null hypothesis could be rejected at P<0.05. All statistical analyses were performed using SPSS for Windows version 19.0 (SPSS Inc., Chicago, Illinois) and STATA 11.2 (STATA Corp., Texas).
Results
A total of 153 subjects were studied. Thirty patients had angiographically documented CAD, but preserved left-ventricular ejection fraction by angiography and no evidence for diastolic heart failure. One hundred and twenty-three patients had a documented history of chronic heart failure (CHF) for at least 3 months. The median duration of heart failure symptoms was 66 months (IR: 35–111 months). The clinical characteristics of the 2 study populations are summarized in Table 1. Importantly, there were no significant differences between the CHF and the CAD group, except for symptoms and quantitative measures (left-ventricular ejection fraction, New York Heart Association-class, Nt-pro-BNP) of heart failure, as well as use of diuretics and digitalis, and hemoglobin levels.

Bone Marrow Plasma Levels of RANKL and OPG
As illustrated in Figure 1A, patients with CHF had significantly (P<0.001) elevated bone marrow plasma levels of RANKL (24.6±17.6 ng/mL) compared with patients with CAD, but preserved left-ventricular function (9.7±8.7 ng/mL). On multivariate linear regression analysis, parameters of severity and duration of heart failure (CHF duration in months, New York Heart Association-class, NT-pro-BNP serum levels) and gender were significant independent determinants of elevated bone marrow plasma RANKL levels (Table 2).

RANKL bone marrow plasma levels were positively correlated with NT-pro-BNP serum levels (r=0.49; P<0.001; n=145). There was no statistically significant (P=0.899) difference in RANKL bone marrow plasma levels between patients with ischemic (24.7±17.7 ng/mL; n=108) compared with nonischemic (24.1±17.6 ng/mL; n=15) cause of heart failure.

Bone marrow plasma levels of OPG were slightly, but nonsignificantly elevated (P=0.123) in patients with CHF (2860.7±1608.3 pg/mL; n=123) compared with patients with CAD, but preserved left-ventricular function (2395.8±828.5 pg/mL; n=30) (Figure 1B). On multivariate analysis, NT-pro-BNP as a marker of the severity of heart failure and serum-creatinine levels remained as independent predictors of elevated OPG bone marrow plasma levels (Table 3).

OPG levels were similar (P=0.822) in patients with ischemic (2888.5±1663.3 pg/mL; n=108) and nonischemic heart failure (2684.5±1220.9 pg/mL; n=15). Importantly,

Table 1. Baseline Characteristics of the Study Population

<table>
<thead>
<tr>
<th>Factor</th>
<th>CAD</th>
<th>CHF</th>
<th>P value (*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>30</td>
<td>123</td>
<td>—</td>
</tr>
<tr>
<td>Age, y</td>
<td>58.90±13.59</td>
<td>64.22±10.50</td>
<td>NS</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>25/5</td>
<td>102/21</td>
<td>NS</td>
</tr>
<tr>
<td>CAD, n/ %</td>
<td>30/100, %</td>
<td>108/87.8, %</td>
<td>NS</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>53.44±8.47</td>
<td>28.90±9.04</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Duration of CHF-symptoms, mos</td>
<td>—</td>
<td>83.8±66.6**</td>
<td>ND</td>
</tr>
<tr>
<td>NYHA functional class</td>
<td>1.67±0.75</td>
<td>2.58±0.68</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Canadian cardiovascular society grading score (CCS)</td>
<td>2.23±1.57</td>
<td>1.76±1.25</td>
<td>NS</td>
</tr>
<tr>
<td>Body mass index (BMI)</td>
<td>29.42±3.00(n=26)</td>
<td>27.8±3.55(n=95)</td>
<td>NS</td>
</tr>
<tr>
<td>Smoking, n/ %</td>
<td>13/44.8, %(n=29)</td>
<td>29/24.4, %(n=119)</td>
<td>0.023</td>
</tr>
<tr>
<td>Diabetes mellitus, n/ %</td>
<td>17/56.7, %</td>
<td>54/43.9, %</td>
<td>NS</td>
</tr>
<tr>
<td>HBA1C, %</td>
<td>7.0±1.4</td>
<td>6.4±1.3</td>
<td>NS</td>
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<tr>
<td>Serum-Creatinine, mg/dL</td>
<td>1.1±0.2</td>
<td>1.2±0.3 (n=121)</td>
<td>NS</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>42.2±3.0</td>
<td>42.2±3.6</td>
<td>NS</td>
</tr>
<tr>
<td>Hemoglobin, g/dL</td>
<td>13.9±1.4</td>
<td>12.6±1.89</td>
<td>NS</td>
</tr>
<tr>
<td>NT-pro-BNP, pg/mL</td>
<td>255.44±199.59(n=25)</td>
<td>2178.09±2048.44(n=120)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>hsCRP, mg/dL</td>
<td>0.38±0.36</td>
<td>0.40±0.41</td>
<td>NS</td>
</tr>
<tr>
<td>Statin, n/ %</td>
<td>22/73.3, %</td>
<td>90/73.8, %(n=122)</td>
<td>NS</td>
</tr>
<tr>
<td>ACE-I / ARB, n/ %</td>
<td>23/76.7, %</td>
<td>111/91.0, %(n=122)</td>
<td>NS</td>
</tr>
<tr>
<td>β-blocker, n/ %</td>
<td>22/73.3, %</td>
<td>88/71.5, %</td>
<td>NS</td>
</tr>
<tr>
<td>Thiazide diuretic, n/ %</td>
<td>9/30.0, %</td>
<td>49/44.5, %(n=110)</td>
<td>NS</td>
</tr>
<tr>
<td>Loop diuretic, n/ %</td>
<td>8/26.7, %</td>
<td>81/65.9, %</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Aldosterone antagonist, n/ %</td>
<td>1/3.4, %(n=29)</td>
<td>65/53.3, %(n=122)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Digitalis, n/ %</td>
<td>3/10.0 , %</td>
<td>47/38.5, %(n=122)</td>
<td>0.003</td>
</tr>
<tr>
<td>Amiodarone, n/ %</td>
<td>3/10.0 , %</td>
<td>22/17.9, %</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data are presented as mean±SD. Plus-minus values are mean±SD. CAD indicates coronary artery disease, LVEF, left-ventricular ejection fraction; CHF, chronic heart failure; NYHA, New York Heart Association, NT-pro-BNP, N-terminal pro-Brain natriuretic peptide; CRP, C-reactive protein; ACE-I, angiotensin-converting enzyme inhibitor; ARB, angiotensin-receptor blocker; ND, not determined; NS, not significant.

*P-value for comparison of CAD and CHF patients.
**median 66 mos (IR: 35–111 mos).
in the group of patients with CHF, there was no correlation ($r=-0.004; P=0.965$) between bone marrow plasma levels of RANKL and OPG.

Because local biological activity of RANKL may depend on how much of the protein is blocked by OPG, we determined the ratio between RANKL and OPG in bone marrow plasma. As illustrated in Figure 1C, the ratio of RANKL/OPG in bone marrow plasma was significantly ($P=0.003$) higher in patients with CHF (11.0±10.4) compared with patients with CAD, but preserved left-ventricular function (4.6±4.4).

To investigate whether RANKL levels are selectively increased in the bone marrow environment, we additionally determined the plasma levels of RANKL in peripheral blood samples in a subgroup of 60 patients, in whom plasma specimens obtained from peripheral blood were available for analysis, and determined the ratio between bone marrow and peripheral blood plasma levels.

The ratio of bone marrow to peripheral blood levels of RANKL was significantly ($P=0.02$) increased in the group of patients with CHF (9.47±9.55; $n=47$) compared with the group of patients with CAD (2.10±1.85; $n=13$).

The increase in the ratio of bone marrow to peripheral blood plasma levels was exclusively due to the elevated bone marrow plasma levels, whereas peripheral blood plasma levels of RANKL did not differ between the groups (CAD-group: 4.49±3.79 ng/mL, $n=13$; CHF-group: 4.66±5.83 ng/mL, $n=47$; $P=0.919$) documenting a selective increase in RANKL concentrations in the bone marrow environment in patients with CHF.

Thus, the bone marrow plasma of patients with CHF is characterized by excessively elevated levels of RANKL, which are not counteracted by comparably increased OPG levels, indicating an imbalance of the cytokines-mediating bone remodeling within the bone marrow.

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**Figure 1.** A, Bone marrow plasma receptor activator of NF-kB ligand (RANKL) levels (ng/mL) in the 2 different study groups. B, Bone marrow plasma osteoprotegerin (OPG) levels (pg/mL) in the 2 different study groups. C, Ratio RANKL/OPG in the bone marrow plasma of the 2 different study groups. Values are means (±SEM). CHF indicates congestive heart failure.
Systemic Levels of PTH

Serum CTX levels as a quantitative marker of bone turnover were significantly elevated ($P=0.014$) in patients with CHF (0.43±0.20 ng/mL; n=55) compared with patients with CAD and preserved left-ventricular function (0.29±0.08 ng/mL; n=13). Importantly, as illustrated in Figure 2, serum levels of CTX were directly correlated with bone marrow plasma levels of RANKL in the group of patients with CHF ($r=0.6; P<0.001, n=55$). Moreover, the correlation between serum levels of CTX with bone marrow plasma levels of RANKL was similarly strong for male ($r=0.5; P=0.001, n=40$) and female ($r=0.8; P<0.0001; n=15$) patients. Finally, serum levels of CTX were also correlated with the ratio of RANKL/OPG in the bone marrow plasma ($r=0.43; P=0.001; n=55$). Thus, elevated levels of the bone resorption stimulating RANKL within the bone marrow environment in patients with CHF are directly associated with an increase in a systemic quantitative biochemical marker of bone turnover.

**Table 2. Univariate and Multivariate Relationships of RANKL Bone Marrow Plasma Levels and Clinical Characteristics**

<table>
<thead>
<tr>
<th></th>
<th>Univariate Analysis</th>
<th>Multivariate Analysis*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\beta^*$</td>
<td>95% CI</td>
</tr>
<tr>
<td>CAD</td>
<td>-2.63</td>
<td>-11.91; 6.65</td>
</tr>
<tr>
<td>CHF</td>
<td>14.86</td>
<td>8.32; 21.39</td>
</tr>
<tr>
<td>CHF duration</td>
<td>0.09</td>
<td>0.05; 0.13</td>
</tr>
<tr>
<td>NYHA-Class</td>
<td>10.07</td>
<td>6.76; 13.37</td>
</tr>
<tr>
<td>Ejection fraction</td>
<td>-0.41</td>
<td>-0.61; -0.20</td>
</tr>
<tr>
<td>Gender</td>
<td>8.66</td>
<td>1.44; 15.88</td>
</tr>
<tr>
<td>Age</td>
<td>0.21</td>
<td>-0.03; 0.45</td>
</tr>
<tr>
<td>Smoking (n=148)</td>
<td>-1.24</td>
<td>-4.55; 2.07</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>-5.31</td>
<td>-10.79; 0.16</td>
</tr>
<tr>
<td>Serum-Creatinine (n=151)†</td>
<td>0.09</td>
<td>-0.98; 1.16</td>
</tr>
<tr>
<td>NT-pro BNP (n=145)§</td>
<td>0.05</td>
<td>0.04; 0.06</td>
</tr>
<tr>
<td>hsCRP</td>
<td>-4.18</td>
<td>-11.04; 2.69</td>
</tr>
<tr>
<td>Statin (n=152)</td>
<td>-0.17</td>
<td>-0.32; -0.02</td>
</tr>
<tr>
<td>Loop diuretic</td>
<td>5.91</td>
<td>0.39; 11.43</td>
</tr>
<tr>
<td>Thiazide diuretic (n=140)§</td>
<td>0.13</td>
<td>-5.89; 6.14</td>
</tr>
<tr>
<td>Aldosterone antagonist (n=152)§</td>
<td>1.86</td>
<td>0.52; 3.21</td>
</tr>
<tr>
<td>Digitals (n=152)</td>
<td>3.53</td>
<td>-2.32; 9.38</td>
</tr>
<tr>
<td>ACE-I / ARB (n=152)</td>
<td>-2.48</td>
<td>-11.28; 6.31</td>
</tr>
<tr>
<td>$\beta$-blocker</td>
<td>-0.18</td>
<td>-6.33; 5.97</td>
</tr>
</tbody>
</table>

RANKL indicates receptor activator of NF-$\kappa$B ligand; CAD, coronary artery disease; CHF, chronic heart failure; NYHA, New York Heart Association; NT-pro-BNP, N-terminal pro-Brain natriuretic peptide; hsCRP, high-sensitive C-reactive protein; ACE-I, angiotensin-converting enzyme inhibitor; ARB, angiotensin-receptor blocker.  

*Estimated mean difference of RANKL bone marrow plasma levels between groups for categorical variables (Coronary artery disease, Chronic heart failure, NYHA-Class, Gender, Smoking, Diabetes mellitus, Statin, Loop diuretic, Thiazide diuretic, Aldosterone antagonist, Digitals, ACE-I / ARB, $\beta$-blocker); increase of 1 unit for metric variables (CHF duration, Ejection fraction, Age, Serum-Creatinine, NT-pro BNP, hsCRP).  

†Significant predictor variables ($P<0.05$) from univariate analyses were entered in the multivariate model; model fit: $F(9,149)=11.4; P<0.001$.  

‡β for increase of 10 pg/mL.  

§β for increase of 0.1 mg/dL.

**Serum Levels of CTX as Biochemical Marker of Bone Turnover**

As PTH is a key regulator of bone turnover, we also measured serum levels of PTH in peripheral blood samples in a subgroup of 91 subjects. Systemic serum levels of PTH were significantly higher in patients with CHF (60.7±31.9 pg/mL; n=68) compared with patients with CAD, but preserved left-ventricular function (38.8±6.0 pg/mL; n=23; $P=0.017$). However, systemic PTH levels did not correlate with RANKL ($r=-0.04; P=0.63$) bone marrow plasma levels. Moreover, although PTH serum levels were inversely related to the duration of heart failure ($r=-0.39; P=0.006$), there was no association of PTH serum levels with the severity of heart failure as measured either by NT-pro-BNP serum levels ($r=-0.02; P=0.91$) or by left-ventricular ejection fraction ($r=-0.16; P=0.34$). Finally, there was no correlation between serum levels of PTH and serum levels of CTX ($r=-0.13; P=0.54$).

**Bone Marrow Plasma Levels of RANKL and OPG in a Mice Model of Heart Failure**

To confirm these clinical observations without the multiple and potentially confounding comorbidities and pharmacological treatment regimens, we finally employed an experimental model of heart failure and measured bone marrow plasma levels of RANKL and OPG 8 weeks after the induction of large myocardial infarction in mice.

As illustrated in Figure 3A, RANKL bone marrow plasma levels were significantly ($P=0.03$) increased in mice with severely impaired left-ventricular function (fractional shortening<20%) 8 weeks after induction of myocardial infarctions. In contrast, OPG bone marrow plasma levels were similar ($P=0.310$) in mice with and without severely impaired...
left-ventricular function postinfarction (Figure 3B). Thus, the alterations in bone marrow plasma levels of RANKL and OPG in chronic postinfarction mice essentially mimicked the findings observed in patients with CHF.

**Cell Culture Studies**

In order to provide direct mechanistic evidence for elevated RANKL levels to mediate bone resorption in CHF, we assessed the formation of TRAP-positive osteoclasts from mesenchymal cells upon stimulation with bone marrow plasma in cell culture studies required freshly harvested bone marrow plasma, we obtained bone marrow plasma from healthy control subjects and patients with CHF, as there is currently no active clinical protocol allowing for harvesting bone marrow in patients with CAD, but preserved LV function. We incubated human mesenchymal stromal cells with bone marrow plasma obtained from patients with CHF and assessed the formation of Tartrate-Resistant Acid Phosphatase-positive osteoclasts after 7 days of cell culturing. As illustrated in Figure 4, stimulation of mesenchymal stromal cells with bone marrow plasma derived from healthy volunteers.

**Figure 2.** Correlation between bone marrow receptor activator of NF-κB ligand (RANKL) levels (ng/mL) and C-telopeptide of type I collagen (CTX)-serum levels (ng/mL) in the group of heart failure patients.
from patients with CHF was inhibited ($P<0.01$) by simultaneous incubation with a RANKL-specific monoclonal antibody (Figure 4), thus documenting a causal role of RANKL in the bone marrow plasma of patients with CHF to promote catabolic bone remodeling.

**Discussion**

The present study is unique as it for the first time investigates secreted mediators of bone remodeling directly within the bone marrow environment by measuring bone marrow plasma levels in patients with a wide range of chronic systolic heart failure. Our results demonstrate that chronic heart failure is associated with a profound and selective elevation of the bone resorption stimulating RANKL within the bone marrow environment. Elevated RANKL bone marrow plasma levels are directly correlated with systemic serum levels of CTX, a sensitive quantitative biochemical marker of bone turnover. An experimental model of heart failure eliminating potential confounding factors confirmed the clinical results. Finally, bone marrow plasma derived from patients with chronic heart failure profoundly stimulated the formation of osteoclasts, and this effect was blocked by RANKL-specific antibodies, thus providing direct proof-of-concept. Taken together, these data disclose a plausible common pathophysiological mechanism linking chronic heart failure with an increased risk for osteoporotic fractures.

The excessively elevated levels of RANKL selectively in the bone marrow environment are not counterbalanced by a similar increase in OPG, which binds to RANKL and inhibits its bone resorption activity by preventing the interaction with its single cognate receptor RANK. Thus, the bone marrow plasma of patients with chronic heart failure is characterized by an imbalance of the OPG/RANKL axis favoring the catabolic effects of the bone resorption stimulating RANKL. Indeed, in patients with chronic heart failure, serum levels of CTX were directly correlated with bone marrow plasma levels of RANKL, further supporting the pivotal role of RANKL in the bone marrow environment to fine-tune bone homeostasis and remodeling. Finally, the $\approx5$-fold increase of bone marrow plasma levels of RANKL compared with systemically circulating RANKL levels indicates a selective activation of the bone marrow niche to release RANKL in patients with chronic heart failure.
Mechanistically, recent experimental studies have provided convincing evidence that various stress conditions modulate bone remodeling by invoking the activity of bone resorbing osteoclasts as well as by suppressing bone-forming osteoblast. Specifically, adrenergic agonists were shown to indirectly stimulate osteoclast differentiation by increasing RANKL expression and secretion in osteoblasts. Indeed, the close correlation between serum levels of NT-pro-BNP as a marker of neurohumoral activation and bone marrow plasma levels of RANKL observed in the present study supports a link with the neuroendocrine systems to contribute to altered bone remodeling in patients with chronic heart failure.

Although analyses in a large population-based co-twin study revealed that, specifically after heart failure, most of the overall increased rate of hip fractures appears to be explained by genes, the complex pharmacotherapy in patients with chronic heart failure might have influenced bone marrow plasma levels of the RANKL/OPG axis in the present study. Although our comprehensive statistical analysis did not reveal any single pharmacological agent that independently correlates with levels of RANKL within the bone marrow environment, statistical methods are limited in modeling complex interactions between a variety of drugs with opposing actions. However, given the close association between RANKL bone marrow plasma levels and serum NT-pro-BNP as well as serum CTX levels, especially within the patient cohort with chronic heart failure, which received rather homogenous state-of-the-art treatment, it is very unlikely that our results are secondary to different pharmacological treatment regimens in patients with CAD, but preserved left-ventricular function, compared with patients with chronic heart failure. In line with this reasoning, bone marrow plasma levels of RANKL and OPG in the chronic experimental postinfarction heart failure model essentially mimicked the results observed in our patients.

Patients with chronic heart failure are well known to exhibit elevated levels of aldosterone, which may stimulate increased calcium excretion leading to elevated PTH levels, which are further enhanced by loop-diuretics. PTH is a key regulator of mineral metabolism and bone turnover. Although systemic levels of serum PTH levels were elevated in patients with chronic heart failure, there was no correlation of PTH with RANKL bone marrow plasma levels, nor with systemic CTX serum levels, thus making it very unlikely that increased PTH concentrations causally contributed to elevated levels of the bone resorption stimulating RANKL within the bone marrow environment. Nevertheless, we cannot exclude that elevated PTH levels might contribute to increased osteoporotic fractures by other mechanisms in patients with chronic heart failure.

A dysregulated balance of the systemic RANKL/OPG axis has been recently implicated in a variety of conditions causally contributed to elevated PTH concentrations. Although experimental studies in animal models of increased bone resorption repeatedly shown to correlate with parameters of bone resorption, showing a selective, 2-fold increase in RANKL bone marrow plasma levels in animal models of increased bone resorption and decreased bone density, the data of the present study support the hypothesis that increased soluble RANKL in the bone marrow microenvironment may contribute to increased bone loss in patients with chronic heart failure. This conclusion is strongly supported by our observations in cell culture experiments, that bone marrow plasma obtained from patients with chronic heart failure stimulated osteoclast formation in a RANKL-dependent fashion, thus documenting a causal link between RANKL bone marrow plasma levels and catabolic bone remodeling.

Obviously, the present study cannot provide definitive proof for a causal link between chronic heart failure and increased RANKL-mediated bone turnover in vivo. Reduced physical activity might also have contributed to increased bone turnover in patients with heart failure. However, given that osteoporotic fracture itself was shown to be a substantial contributor to all-cause mortality in patients with heart failure, pharmacological targeting of elevated RANKL levels in the bone marrow environment by the recently developed antibody against RANKL might be an attractive common therapeutic target for 2 of the most important public health conditions, namely chronic heart failure and osteoporosis.

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Disclosures
None.

References
Elevated Levels of the Mediator of Catabolic Bone Remodeling RANKL in the Bone Marrow Environment Link Chronic Heart Failure with Osteoporosis

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