Low Serum Ferroxidase I Activity Is Associated With Mortality in Heart Failure and Related to Both Peroxynitrite-Induced Cysteine Oxidation and Tyrosine Nitrination of Ceruloplasmin

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Rationale: Ceruloplasmin antioxidant function is mainly related to its ferroxidase I (FeOXI) activity, which influences iron-dependent oxidative and nitrosative radical species generation. Peroxynitrite, whose production is increased in heart failure (HF), can affect ceruloplasmin antioxidant function through amino acid modification.

Objective: We investigated the relationship between FeOXI and ceruloplasmin tyrosine and cysteine modification and explored in a cohort of patients with HF the potential clinical relevance of serum FeOXI.

Methods and Results: In patients with chronic HF (n=96, 76±9 years; New York Heart Association class, 2.9±0.8) and age-matched controls (n=35), serum FeOXI, FeOXII, ceruloplasmin, nitrotyrosine-bound ceruloplasmin, B-type natriuretic peptide, norepinephrine, and high-sensitivity C-reactive protein were measured, and the patients were followed up for 24 months. Ceruloplasmin, B-type natriuretic peptide, norepinephrine, and high-sensitivity C-reactive protein were increased in HF versus controls. FeOXI was decreased in HF (~20%) and inversely related to nitrotyrosine-bound ceruloplasmin (r, −0.305; P=0.003). In HF, FeOXI lower tertile had a mortality rate doubled compared with middle-higher tertiles. FeOXI emerged as a mortality predictor (hazard ratio, 2.95; 95% confidence intervals [1.29–6.75]; P=0.011) after adjustment for age, sex, hypertension, smoking, sodium level, estimated glomerular filtration rate, and high-sensitivity C-reactive protein. In experimental settings, peroxynitrite incubation of serum samples and isolated purified ceruloplasmin reduced FeOXI activity while increasing ceruloplasmin tyrosine nitrination and cysteine thiol oxidation. Reduced glutathione prevented peroxynitrite-induced FeOXI drop, tyrosine nitrination, and cysteine oxidation; flavonoid(-)-epicatechin, which prevented ceruloplasmin tyrosine nitrination but not cysteine oxidation, partially impeded peroxynitrite-induced FeOXI drop.

Conclusions: Reduced activity of serum FeOXI is associated with ceruloplasmin nitration and reduced survival in patients with HF. Both ceruloplasmin tyrosine nitrination and cysteine thiol oxidation may be operant in vivo in peroxynitrite-induced FeOXI activity inhibition. (Circ Res. 2014;114:1723-1732.)

Key Words: ceruloplasmin ■ heart failure ■ mortality ■ oxidative stress

The progression of heart failure (HF) is associated with aberrant oxygen–derived and nitric oxide–derived free radical production in either quantity or spatial cellular localization.1,2 Ferrous iron(II) is a catalyst in the formation of oxygen-derived and nitric oxide–derived free radicals, and its oxidation to ferric(III) generates the propagation of oxidative and nitrosative stress through the Fenton reaction.3 The ability to oxidize ferrous iron(II) ions to less toxic ferric(III) ions and to inhibit iron-dependent free radical and oxidant generation4 is indicated as ferroxidase activity.5

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Ceruloplasmin, an α2-glycoprotein mainly synthesized by hepatocytes, whose functions include the transport of serum copper,6 the acute phase inflammation reactant, is also
involved in iron metabolism through its ferroxidase activity. In fact, ceruloplasmin represents the main contributor of ferroxidase activity in human plasma and is called ferroxidase because of its almost complete inhibition by azide.

It has been recently reported that high ceruloplasmin circulating levels have an independent prognostic value in stable patients undergoing elective diagnostic cardiac catheterization procedure and are associated with the incidence of HF and mortality in the Atherosclerosis Risk in Communities study population.

In another prospective study, a relationship between serum ceruloplasmin levels and the subsequent development of myocardial infarction in the elderly general population was reported.

In settings of experimental and human HF, increased peroxynitrite generation, from nitric oxide and superoxide or from hemoperoxidases, leads to extensive tyrosine protein nitration. Tyrosine nitration may affect protein structure with a loss, a gain, or no effect on function. Cysteine can also be modified by peroxynitrite, and, in particular, sulfhydryl oxidation can lead to misfolding of proteins and remarkably influence their function.

Ceruloplasmin has been identified among nitrated proteins in patients with acute respiratory distress syndrome and in patients on hemodialysis. It is reported that ceruloplasmin tyrosine nitration affects protein function, resulting in a reduction of ferroxidase activity. In the HF population, no data are available on the relationship between ceruloplasmin, its tyrosine nitration, and on FeOxI activity and even less on the clinical relevance of ceruloplasmin-mediated FeOxI activity. In addition, no information in clinical or experimental settings is available on an involvement of other amino acid changes, such as cysteine thiol oxidation in peroxynitrite-induced FeOxIII activity drop of ceruloplasmin. Based on these premises, we undertook a prospective study on a cohort of stable elderly patients with chronic HF to investigate the prognostic value of ceruloplasmin-mediated FeOxI activity and to evaluate the relationship of ceruloplasmin-mediated FeOxII activity and its tyrosine nitration. We also explored the role of tyrosine nitration and cysteine thiol oxidation in peroxynitrite-mediated ceruloplasmin FeOxI activity inhibition in experimental settings.

**Methods**

### Study Cohort and Follow-Up of Patients

Ninety-six consecutive stable patients with chronic HF, who referred to the HF outpatient Clinic of the Cardiorenal Research Unit of the Department of Clinical and Experimental Medicine of the University Hospital of Parma, were prospectively studied for 24 months. The diagnosis of HF was based on symptoms and clinical signs according to guidelines issued by the European Society of Cardiology and by the American College of Cardiology. The patients had no clinical or laboratory signs of acute infection, rheumatoid or other autoimmune diseases, primary cachectic states (cancer, thyroid disease, severe liver disease, severe chronic lung disease), neuromuscular disorders, myocardial infarction within the previous 20 weeks, diabetes mellitus, or severe chronic renal failure (serum creatinine level >2.0 mg/dL, >177 μmol/L). All patients were clinically stable and on constant therapy ≥8 weeks before entering the study. The study was approved by the University of Parma Ethics Committee, complied with the Declaration of Helsinki, and all participants provided written informed consent.

Thirty-five age-matched healthy subjects were recruited as controls from healthy subjects reporting for a periodical check-up at the cardiovascular prevention Clinic of the same Department. On study entry, a complete medical history, a physical examination, basal laboratory tests (total blood count, including red cell distribution width, serum creatinine, electrolytes, lipid profile), plasma neurohumoral and inflammatory markers determination, an ECG, and an echo-cardiogram were obtained from all patients. Estimated glomerular filtration rate (eGFR) was calculated from the 4-component Model of Disease in Renal Disease equation incorporating age, race, sex, and serum creatinine level: estimated eGFR=186 × (serum creatinine [in milligrams per deciliter]⁻¹.1154 × (age in years))⁻⁰.209. For women, the product of the equation was multiplied by a correction factor of 0.742. All patients were seen ≥4× per year in our HF outpatient clinic or as per their specific clinical need. The primary end point was all-cause mortality: this information was obtained by their general physician and by relatives. Morbidity data expressed as time to first hospital admission, number of hospitalizations for heart failure, and cumulative length of hospital stay during the follow-up period were also recorded.

### Venous Blood Sampling Procedure and Biochemical Assays

Venous samples were obtained after ≥30 minutes of supine rest from an indwelling catheter and collected in polypropylene tubes containing an EDTA buffer (1.5 mg/mL), except for B-type natriuretic peptide (BNP) where a mix of protease inhibitors (phenylmethylsulfonyl fluoride, trypsin inhibitor, and aprotonin 500 U/mL) was added and for serum copper where EDTA was excluded. Fresh serum samples were used to measure FeOxI and FeOxII activities, whereas multiple aliquots of plasma samples were stored at −80°C until assay time for norepinephrine, BNP, free malondialdehyde, total nitrated proteins, ceruloplasmin, nitrotyrosine-bound ceruloplasmin, and total copper. All laboratory measurements were performed without any freeze-thaw cycles of the samples and by investigators blind to the clinical data.

FeOxI and FeOxII activity was measured by ferrous ion as substrate (Fe(II); ferrous ammonium) according to the method of Erel. Briefly, serum was incubated with Fe(II) in 0.45 mol/L acetate buffer (pH 5.8), and the remaining nonoxidized ferrous ions formed a colored complex with the 3-(2-pyridyl)-5,6-bis(2-[5-furylsulfonic acid]-1,2,4-triazine chromogen. The difference in the ferrous ion concentration before and after the reaction corresponded to the amount of Fe(II) oxidized to Fe(III) and directly expressed the total ferroxidase activity of the serum. For FeOxII, the same procedure was applied using serum previously mixed with sodium azide, which instantaneously and completely inhibited FeOxI. FeOxI activity was calculated from the difference between total and FeOxIII activities. Serum concentrations of copper were analyzed by use of atomic absorption spectrophotometry. Intra-assay and interassay values for coefficient of variation were 4.1% and 4.6%, respectively.

Norepinephrine, BNP, free malondialdehyde, and high-sensitivity C-reactive protein (hsCRP) were determined as previously described. Serum ceruloplasmin was evaluated using an immuno-nephelometry kit (N antisemur against human ceruloplasmin,

### Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BNP</td>
<td>B-type natriuretic peptide</td>
</tr>
<tr>
<td>EF</td>
<td>ejection fraction</td>
</tr>
<tr>
<td>eGFR</td>
<td>estimated glomerular filtration rate</td>
</tr>
<tr>
<td>FeOxI</td>
<td>ferroxidase I</td>
</tr>
<tr>
<td>FeOxII</td>
<td>ferroxidase II</td>
</tr>
<tr>
<td>GSH</td>
<td>reduced glutathione</td>
</tr>
<tr>
<td>HF</td>
<td>heart failure</td>
</tr>
<tr>
<td>hsCRP</td>
<td>high-sensitivity C-reactive protein</td>
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<td>IQR</td>
<td>interquartile range</td>
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Peroxynitrite Synthesis and Ex Vivo Experiments on Ceruloplasmin-Related Ferrooxidase Activity

To evaluate the effects of peroxynitrite (0.15 mmol/L) and decomposed peroxynitrite addition on ceruloplasmin-related FeOxI activity and its relationship with ceruloplasmin nitration, separate ex vivo experiments were performed in a subset of controls (n=18). Serum samples (350 μL of fresh serum) from these subjects had FeOxI and II activities measured before and after addition of peroxynitrite and decomposed peroxynitrite prepared as previously described. The samples were incubated for 15 minutes at 37°C with peroxynitrite or with decomposed peroxynitrite (0.15 mmol/L) in phosphate buffer (K₂HPO₄ 0.1 mol/L, 650 μL, pH 7.40); these analyses were performed in triplicate. The concentration of peroxynitrite used in the present study is of pathophysiological relevance. Separate in vitro experiments (n=10), using lyophilized ceruloplasmin (Sigma-Aldrich Chemical Co, St Louis, MO) dissolved at 50 μg/4.5 U in K₂HPO₄ 0.1 mol/L, pH 7.40, have been performed, and FeOxI and II activities were measured before and after addition of peroxynitrite (0.15 mmol/L in phosphate buffer) and decomposed peroxynitrite for 15 minutes at 37°C. Ferrooxidase activities were also measured in the presence of reduced glutathione (GSH, 850 μmol/L, Acros Organic, Thermo-Fisher Scientific) or (-)-epicatechin (50 μmol/L; Sigma-Aldrich Chemical Co), a flavanol known for its ability to protect against nitration but not from oxidation induced by peroxynitrite. GSH and (-)-epicatechin at these concentrations, in previous in vitro experiments, induced similar reductions in nitration of free tyrosine after peroxynitrite exposure. Standard solutions of (-)-epicatechin were prepared in dimethyl sulfoxide and stored at −20°C; after dilution, the final concentration of dimethyl sulfoxide was 0.01%.

Ceruloplasmin Immunoprecipitation and Immunoblotting for Nitrotyrosine and Cysteine Thiol Oxidation

For immunoprecipitation, we used a polyclonal antihuman anti-ceruloplasmin antibody (ABIN680108 antibodies-online GmbH, Aachen, Germany) that was cross-linked to Dynabeads protein A (Dynal Biotech, Oslo, Norway). Immunoprecipitated ceruloplasmin was then tested for nitrotyrosine with immunoblotting and ELISA assays (ELISA assay Kit; Oxis Research International Inc, Foster City, CA) as previously described. Nitrotyrosine-bound ceruloplasmin was evaluated in serum from patients with HF and controls but also in ex vivo experiments on control serum (n=18) after incubation of peroxynitrite or decomposed peroxynitrite and in in vitro experiments (n=10) after peroxynitrite or decomposed peroxynitrite incubation of isolated purified ceruloplasmin in a phosphate buffer bath. In addition to nitrotyrosine, the evaluation of cysteine thiol oxidation after peroxynitrite or decomposed peroxynitrite incubation was performed on isolated purified ceruloplasmin in a phosphate buffer bath. To detect cysteine thiol status, we labeled untreated and peroxynitrite or decomposed peroxynitrite-treated ceruloplasmin free thiol groups with iodoacetamidofluorescein. Iodoacetamidofluorescein-labeled thiol groups were detected using 1 mg/mL mouse antifluorescein-Oregon Green (Molecular Probes) as previously described. For detailed experimental methods on ceruloplasmin immunoprecipitation and immunoblotting on ceruloplasmin nitrotyrosine and cysteine thiol oxidation detection, see the Online Data Supplement.

Data Analysis

Values are presented as mean±SD. Comparisons of the baseline characteristic variables among the tertiles of FeOxI activity were made with 1-way ANOVA model followed by the Bonferroni post hoc test. Univariate and multivariate analyses using Cox regression techniques were performed to examine the relationship between variables and mortality after 2-year follow-up period. Variables accepted as being of prognostic value in HF were included in the Cox model (age, sex, hypertension, smoking status, plasma sodium level, eGFR, hsCRP, BNP). Four models of analysis including different covariates are reported in Table 3. Statistical comparisons were performed by Kaplan–Meier survival curves to analyze the cumulative event rate linked to tertiles of FeOxI activity (tertile 1, FeOxI ≤336 UI/L; tertile 2, 336<FeOxI<432 UI/L; tertile 3, FeOxI ≥432 UI/L). Data on time to first hospitalization, number of hospitalizations for heart failure, and cumulative length of hospital stay are given as median and interquartile range (IQR). All statistical analyses were performed using SPSS for Windows version 18.0 (SPSS Inc). Relationships between parameters such as FeOxI, ceruloplasmin, nitrotyrosine-bound ceruloplasmin, total nitrated protein, hsCRP, tumor necrosis factor-α, BNP, malondialdehyde, and red cell distribution width were analyzed by linear regression analysis using Pearson correlation coefficients. The D’Agostino–Pearson normality test was passed for all parameters, except hsCRP that was log transformed to create a normal distribution. P≤0.05 was considered statistically significant.

Results

Ninety-six patients with HF met the inclusion criteria and agreed to participate in the study (47 women and 49 men). Their mean age was 76±9 years, and their mean New York Heart Association functional class was 2.9±0.8 (class II/III/IV: 38/31/27). The baseline features are outlined in Table 1, and clinical parameters were compared with age-matched controls (n=35). Patients with HF had a lower ejection fraction (EF, 42±11%) and eGFR (46±16 mL/min) compared with controls. Setting at 45%, the cutoff for EF, 57 (59%) patients with HF had a reduced EF (34±8%), and 39 (41%) patients with HF had a preserved EF (53±5%). HF cause was ischemic in origin in 81% of the patients, and 61% of them had hypertension. Eighty-one percent of the patients were treated with either an angiotensin-converting enzyme inhibitor or an angiotensin-II receptor-1 antagonist, 66% with β-adrenergic blocker, 34% with aldosterone receptor antagonist, 87% with diuretics, and 30% with digoxin. Patients with HF showed significant higher plasma levels of norepinephrine, hsCRP, free malondialdehyde, ceruloplasmin, nitrotyrosine-bound ceruloplasmin, and total nitrated proteins as compared with controls (Table 1). Total serum copper levels were higher in patients with HF compared with those in controls (21.63±6.77 versus 16.45±4.87 μmol/L; P<0.01).

FeOxI activity was significantly reduced in patients with HF versus controls (−20%; P<0.01; Table 1), whereas no difference in FeOxII was observed. When the comparison of FeOxI activity was made by dividing the patients on the basis of their New York Heart Association class, a significant reduction of FeOxI was observed: New York Heart Association class IV patients compared with class II patients (P<0.05) and with controls (P<0.01) as well as New York Heart Association class III patients compared with controls (P<0.01) had a lower FeOxI activity (Figure 1A). FeOxI activity was significantly reduced in HF with reduced EF versus those with preserved EF (354±21 UI/L versus 426±19 UI/L; P=0.014).

In Table 2, the baseline characteristics of patients with HF are depicted separately for each tertile of FeOxI activity. No
differences were observed among tertiles with regard to age, body mass index, systolic blood pressure, heart rate, hemoglobin, eGFR, hsCRP, free malondialdehyde, ferritin, and total nitrated proteins (Table 2), and total copper levels.

There were no significant differences in the medical therapy among tertiles of patients. Tertile 1 (FeOxI activity ≤336 UI/L) had lower diastolic blood pressure, lower EF, higher BNP, and nitrated ceruloplasmin compared with tertile 3 (FeOxI activity >432 UI/L). No significant differences were found in plasma norepinephrine levels among ferroxidase tertiles even if a trend was observed toward higher levels in tertiles 1 and 2 versus tertile 3 (P=0.053; Table 2). Serum ceruloplasmin was higher in tertile 1 as compared with tertile II but not different from tertile III (Table 2). Follow-up was complete in all patients. After 24 months of follow-up, 40 patients (41%) of the HF group died: 20 (64%) patients died in tertile 1, 11 (33%) in tertile 2, and 9 (28%) patients in tertile 3 of FeOxI activity. As shown in Table 3, multivariate Cox regression analysis revealed that FeOxI activity was an independent predictive factor for death occurrence (hazards ratio, 2.95; 95% confidence intervals [1.29–6.75]; P=0.011) even after adjustment of the model for clinical variables including age, sex, hypertension, smoking habit, plasma sodium level, eGFR, and RDW, red cell distribution width.

Figure 1A. Boxplots of serum ferroxidase I activities in controls (n=35) and patients with heart failure (HF; New York Heart Association classes II, n=38; III, n=31; IV n=27). One-way ANOVA (P<0.001) showed a significant difference among the groups (Class IV and III vs controls, †P<0.01; Class IV vs Class II, ‡P<0.05). B and C, Scatterplots of serum ferroxidase I activity against ceruloplasmin (B) and nitrotyrosine-bound ceruloplasmin (C) in patients with HF. r=Pearson correlation coefficient.
Cumulative length of hospital stay was higher in FeOxI tertile 1 versus tertile 3 (median, 32 days [IQR, 18–56 days] for tertile 1 versus 26 days [IQR, 16–33 days] for tertile 3; \( P =0.025 \)) but not significant versus tertile 2 (tertile 2: median 23 days [IQR, 12–45 days]; \( P =0.152 \)). No significant differences among FeOxI tertiles were found on number of hospitalizations in 24 months (median, 3 days [IQR, 2–4 days] for tertile 1; median, 2 days [IQR, 1–3 days] for tertile 3; \( P =0.467 \)).

In patients with HF, no correlation was found between serum FeOxI activity and ceruloplasmin (\( r, 0.016; \ P =0.872; \) Table 2).

### Table 2. Characteristics of Patients With Heart Failure Divided for Ferroxidase I Activity Tertiles

<table>
<thead>
<tr>
<th>Tertile</th>
<th>(n=31) FeOx≤336 UI/L</th>
<th>Tertile 2 (n=33) 336&lt;FeOx&lt;432 UI/L</th>
<th>Tertile 3 (n=32) FeOx≥432 UI/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>77±9</td>
<td>76±8</td>
<td>75±10</td>
</tr>
<tr>
<td>Sex, male</td>
<td>21</td>
<td>18</td>
<td>10</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>23.9±2.8</td>
<td>23.4±3.8</td>
<td>25.3±3.8</td>
</tr>
<tr>
<td>Systolic BP, mmHg</td>
<td>127±24</td>
<td>128±22</td>
<td>136±17</td>
</tr>
<tr>
<td>Diastolic BP, mmHg</td>
<td>70±15</td>
<td>72±13</td>
<td>80±14*</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>72±13</td>
<td>72±11</td>
<td>71±13</td>
</tr>
<tr>
<td>Ischemic heart disease</td>
<td>24 (77)</td>
<td>27 (82)</td>
<td>23 (72)</td>
</tr>
<tr>
<td>Hypertensive heart disease</td>
<td>9 (29)</td>
<td>10 (30)</td>
<td>19 (59)</td>
</tr>
<tr>
<td>Idiopathic heart disease</td>
<td>3 (10)</td>
<td>0 (0)</td>
<td>1 (3)</td>
</tr>
<tr>
<td>NYHA class (II, III, IV)</td>
<td>6, 12, 13</td>
<td>13, 8, 12</td>
<td>18, 11, 3</td>
</tr>
<tr>
<td>Ejection fraction, %</td>
<td>39±13</td>
<td>40±12</td>
<td>47±9*</td>
</tr>
<tr>
<td>Hemoglobin, g/dL</td>
<td>13.1±1.1</td>
<td>12.8±1.4</td>
<td>13.1±1.2</td>
</tr>
<tr>
<td>RDW, %</td>
<td>14.1±1.1</td>
<td>13.2±0.7*</td>
<td>13.2±0.9*</td>
</tr>
<tr>
<td>eGFR, mL/min</td>
<td>44.1±12.5</td>
<td>43.5±14.0</td>
<td>50.4±20.3</td>
</tr>
<tr>
<td>hsCRP, mg/dL</td>
<td>5.39±7.94</td>
<td>6.10±7.61</td>
<td>2.90±6.81</td>
</tr>
<tr>
<td>Tumor necrosis factor-α, pg/mL</td>
<td>5.25±1.59</td>
<td>4.50±2.38</td>
<td>3.70±1.61*</td>
</tr>
<tr>
<td>Norepinephrine, pg/mL</td>
<td>445±191</td>
<td>433±247</td>
<td>339±122</td>
</tr>
<tr>
<td>BNP, pg/mL</td>
<td>282.8±179.3</td>
<td>175.8±146.1*</td>
<td>113.5±78.8*</td>
</tr>
<tr>
<td>Free malondialdehyde, μmol/L</td>
<td>0.42±0.14</td>
<td>0.42±0.13</td>
<td>0.35±0.11</td>
</tr>
<tr>
<td>Ferritin, ng/mL</td>
<td>2545±568†</td>
<td>2233±468</td>
<td>2485±493</td>
</tr>
<tr>
<td>Ferritin, mmol/L</td>
<td>120.5±79.3</td>
<td>154.3±110.9</td>
<td>132.2±68.6</td>
</tr>
<tr>
<td>Ferridoxin II activity, UI/L</td>
<td>13.7±2.9</td>
<td>14.1±5.4</td>
<td>14.5±4.6</td>
</tr>
<tr>
<td>Total nitrated proteins, nmol/L</td>
<td>390.22±111.86</td>
<td>390.22±83.16</td>
<td>354.26±83.67</td>
</tr>
<tr>
<td>Nitrotyrosine-bound ceruloplasmin, nmol/L</td>
<td>16.05±11.80</td>
<td>10.55±7.76*</td>
<td>9.24±6.40*</td>
</tr>
</tbody>
</table>

Data are reported as mean±SD; numbers in parentheses are percentages. BMI indicates body mass index; BNP, B type natriuretic peptide; BP, blood pressure; eGFR, estimated glomerular filtration rate; FeOx, ferroxidase; hsCRP, high-sensitivity C-reactive protein; NYHA, New York Heart Association; and RDW, red cell distribution width.

\*P<0.05 vs tertile 1; †P<0.05 vs tertile 2.

Cumulative length of hospital stay was higher in FeOxI tertile 1 versus tertile 3 (median, 32 days [IQR, 18–56 days] for tertile 1 versus 26 days [IQR, 16–33 days] for tertile 3; \( P =0.025 \)) but not significant versus tertile 2 (tertile 2: median 23 days [IQR, 12–45 days]; \( P =0.152 \)).

In patients with HF, no correlation was found between serum FeOxI activity and ceruloplasmin (\( r, 0.016; \ P =0.872; \) Table 2).

### Table 3. Unadjusted and Adjusted Cox Proportional Hazard Models for Mortality According to Tertiles of FeOxI Activity

<table>
<thead>
<tr>
<th>Models</th>
<th>Unadjusted</th>
<th>Adjusted for clinical variables*</th>
<th>Adjusted for clinical variables* and hsCRP</th>
<th>Adjusted for clinical variables* and BNP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Events at 24 mo, n (%)</td>
<td>9 (28)</td>
<td>11 (33)</td>
<td>20 (64)</td>
<td></td>
</tr>
<tr>
<td>Upper FeOxTertile</td>
<td>HR (95% CI)—( P ) value</td>
<td>1.0</td>
<td>1.27 (0.53–3.07) – 0.592</td>
<td>3.65 (1.66–8.04) – &lt;0.001</td>
</tr>
<tr>
<td>Middle FeOxTertile</td>
<td>HR (95% CI)—( P ) value</td>
<td>1.0</td>
<td>0.89 (0.35–2.27) – 0.805</td>
<td>3.23 (1.37–7.64) – 0.009</td>
</tr>
<tr>
<td>Lower FeOxTertile</td>
<td>HR (95% CI)—( P ) value</td>
<td>1.0</td>
<td>0.87 (0.34–2.21) – 0.764</td>
<td>2.95 (1.28–6.75) – 0.011</td>
</tr>
</tbody>
</table>

\*The model was adjusted for clinical variables includes age, sex, hypertension, smoking habit, plasma sodium level, and estimated glomerular filtration rate.
Figure 1B), whereas a significant inverse linear relationship was observed between FeOx I and nitrotyrosine-bound ceruloplasmin (r, −0.305; P=0.003; Figure 1C), total nitrotyrosine (r, −0.289; P=0.007), free malondialdehyde (r, −0.246; P=0.015), hsCRP (r, −0.245; P=0.017), tumor necrosis factor-α (r, −0.288; P=0.009), BNP (r, −0.427; P<0.001), and red cell distribution width (r, −0.327; P=0.001).

When patients with HF were dichotomized on the basis of EF, those with preserved EF had significantly lower ceruloplasmin levels (2265±419 versus 2523±563 nmol/L; P=0.017) as well as hsCRP (2.20±5.71 versus 6.61±8.09 mg/dL; P=0.004) as compared with patients with HF with reduced EF, and a trend in reduction was found for nitrotyrosine-bound ceruloplasmin (9.27±7.53 versus 13.31±10.13 nmol/L; P=0.068). Mean FeOxI activity in HF with reduced EF was lower than in those with preserved EF (355±144 versus 426±129 UI/L; P=0.015). At the end of follow-up, 27 of 57 (47%) patients died in the group with reduced EF compared with 13 of 39 (33%) patients with preserved EF. Median time to first hospitalization was shorter in HF with reduced EF (31 days [IQR, 18–52 days]) as compared with 19 days [IQR, 11–29 days]; P=0.01). A negative relationship was observed in the patients with HF with reduced EF between FeOxI and hsCRP (r, −0.325; P=0.013; n=57) but not in those with preserved EF (r, 0.206; P=0.201; n=39). Both preserved and reduced EF patient groups with HF showed a negative relationship between FeOxI and nitrotyrosine-bound ceruloplasmin (preserved EF r, −0.337; P=0.035; n=39 and reduced EF r, −0.256; P=0.054; n=57).

Peroxynitrite Effect on FeOx I Activity, Ceruloplasmin Tyrosine Nitration, and Cysteine Oxidation

After immunoprecipitation, ceruloplasmin from plasma of patients with HF and controls was analyzed by Western blot, and 2 bands at about Mr 140,000 were observed (Figure 3A). Figure 3B shows typical results of immunoblotting assays of eluates from patients with HF and controls tested against anti-nitrotyrosine antibody after ceruloplasmin immunoprecipitation. ELISA assays showed that patients with HF had higher plasma nitrotyrosine-bound ceruloplasmin levels (+203%; P<0.01) compared with controls (Table 1).

In ex vivo experiments, the effects of peroxynitrite on FeOxI and II activities were evaluated in plasma from a subset of controls (n=18 of 35): peroxynitrite addition caused a significant drop in FeOxI activity (from 448±111 to 238±68 UI/L, −47%, Figure 4A, P<0.01) with a concomitant increase in nitrotyrosine-bound ceruloplasmin (from 5.92±2.06 to 21.98±10.49 nmol/L, +371%, Figure 4B, P<0.01). Decomposed peroxynitrite addition did not induce any changes in FeOxI activity or in nitrotyrosine-bound ceruloplasmin levels (Figure 4A and 4B).

The effects of peroxynitrite and decomposed peroxynitrite were also verified on purified and lyophilized ceruloplasmin dissolved in phosphate buffer. The experiments showed that addition of peroxynitrite induced a 77% reduction in FeOxI activity (Figure 5A) associated with an increase in nitrotyrosine-bound ceruloplasmin (3.3× the basal values, P<0.001, Figure 5B). We also investigated the effects of peroxynitrite on cysteine thiol groups of ceruloplasmin. Peroxynitrite treatment of ceruloplasmin decreased iodoacetamidofluorescein labeling of isolated ceruloplasmin (−64%, P<0.001), whereby indicating an increased cysteine thiol oxidation after peroxynitrite incubation (Figure 5C). GSH at a concentration of 850 μmol/L completely prevented peroxynitrite-induced FeOxI drop, tyrosine nitration, and cysteine thiol modification (Figure 5A–5C). In the presence of (-)-epicatechin, peroxynitrite-induced FeOxI activity drop was partially prevented (Figure 5A). Ceruloplasmin tyrosine nitration was completely prevented (Figure 5B), whereas iodoacetamidofluorescein labeling was still decreased, indicating no effects of (-)-epicatechin on cysteine oxidation.
thiol oxidation after peroxynitrite incubation (Figure 5C). When dimethyl sulfoxide, the vehicle of (-)-epicatechin was tested at the dilution used in our experiments, no changes in FeOxI activity, tyrosine nitration, or in iodoacetamido-fluorescein labeling were observed after peroxynitrite incubation (Figure 5A–5C). The result of the immunoblotting assay for iodoacetamidofluorescein labeling of cysteine thiol of isolated and lyophilized ceruloplasmin dissolved in phosphate buffer before and after peroxynitrite incubation in presence and absence of GSH and (-)-epicatechin is depicted in Online Figure I.

Discussion

Three main results arise from this study on a cohort of patients with chronic HF and on in vitro experimental observations. First, we confirm the increase of circulating nitrated proteins in patients with HF compared with controls, as a result of increased peroxynitrite generation. Second, we found that an elevation of nitrotyrosine-bound ceruloplasmin levels in patients with advanced HF was associated with a lower serum FeOxI activity with a close inverse relationship. In support of their close link, we have demonstrated in ex vivo and in vitro experiments that peroxynitrite induces ceruloplasmin tyrosine nitration and cysteine thiol oxidation, and that these amino acid residue changes result in a significant reduction of ceruloplasmin-related FeOxI activity.

Third, in our cohort of patients with moderate-to-severe HF, the reduced FeOxI activity was associated with a significant increase in 2-year mortality. To our knowledge, this is the first study reporting an independent prognostic value for FeOxI activity in patients with HF even after adjustment for clinical and biochemical variables.

All these findings deserve specific comments. First, the evidence for protein nitration in vivo is abundant and solid in both physiological and pathological conditions. In particular, in HF, both experimental and human, nitrotyrosine protein accumulation reflects a disruption in the balance between oxygen and nitric oxide–derived oxidant formation and antioxidant defense mechanisms. In the past decade, several experimental studies in HF, but only few in humans, suggested a potential pathogenetic link between progressive deterioration of heart function and increased protein nitration, although they rarely identified which protein was nitrated. The results of our study fully agree with those reported by others showing that severely diseased patients with HF express the highest levels of plasma nitrated proteins. We go further by indicating that ceruloplasmin in patients with HF is identified as one of the plasma-nitrated protein. Protein nitrotyrosine formation has been claimed as a footprint for peroxynitrite generation, but only recently specific nitrated proteins have been identified as having a direct pathogenic role in heart function deterioration.
peroxynitrite generation but alternative mechanisms of nitration have been shown to take place in vivo, involving the generation of the NO$_2$ radical by myeloperoxidase and eosinophil peroxidase in the presence of hydrogen peroxide.\textsuperscript{16,28} Tyrosine nitration is strongly enhanced by the presence of transition metals, in particular iron and copper, because of the formation of secondary radicals at the metal center reacting to NO$_2$.\textsuperscript{20} Therefore, serum FeOxI activity that regulates the balance between toxic ferrous iron and its nontoxic ferric form represents a fundamental mechanism of protection from iron-mediated free radical injury. Serum ceruloplasmin acts as an antioxidant in vivo by binding copper and preventing free copper ions from catalyzing oxidative damage and in particular by its FeOxI activity.\textsuperscript{4,7} We found a progressive decline of serum FeOxI activity while increasing the severity of heart failure along with a parallel increase of nitrotyrosine-bound ceruloplasmin. Nitrotyrosine-bound ceruloplasmin was identified by immunoprecipitation, immunoblot, and ELISA assays; however, we did not investigate which tyrosine residue (ceruloplasmin has 6% of tyrosine residues in its sequence of 1046 amino acids) underwent a nitration process and which has the most important role in FeOxI activity reduction. Hence, we used experimental settings to investigate the effects of peroxynitrite on ceruloplasmin-related ferroxidase activity and tyrosine and cysteine residue modifications.

Our observations provide direct evidence that peroxynitrite is a potent inhibitor of ceruloplasmin-related FeOxI activity. Decomposed peroxynitrite had null effect on FeOxI activity. We also demonstrated that peroxynitrite effects are related not only to modified tyrosine but also involve cysteine residues. This is the first report showing an involvement of both cysteine thiol oxidation and tyrosine nitration in the regulation of ceruloplasmin-related FeOxI activity and that GSH, which inhibits both peroxynitrite-induced ceruloplasmin tyrosine nitration and cysteine thiol oxidation, completely prevent FeOxI activity drop. In the presence of (-)-epicatechin, a flavonoid found in cocoa, grapes, and green tea, which impedes tyrosine nitration but not cysteine thiol oxidation, a partial drop of FeOxI activity was observed after peroxynitrite incubation, indicating that both cysteine and tyrosine are necessary and contribute to full FeOxI activity of ceruloplasmin. Unfortunately, we did not investigate which residues in the ceruloplasmin sequence are modified nor the mechanisms by which the interactions of cysteine and tyrosine residues are important in FeOxI activity. It should be noted from the results obtained in in vitro and ex vivo experiments and in clinical samples that a clear and definite causal link cannot be drawn between peroxynitrite-induced reduction of FeOxI activity and ceruloplasmin cysteine thiol oxidation and tyrosine nitration in patients with HF. Our observations may suggest that these mechanisms of post-translational modification of ceruloplasmin may be also operant in vivo.

In the present study, we observed that in a cohort of elderly stable patients with chronic moderate-to-severe HF, a lower serum FeOxI activity is associated with higher incidence of all-cause mortality in a 2-year follow-up period. This observation has never been reported before and is consistent with the concept that as HF deteriorates, declining FeOxI activity is related to increased nitrotyrosine-bound ceruloplasmin and could contribute to worse outcomes in patients with HF. Interestingly, the predictive mortality performance of FeOxI activity was maintained after the model was adjusted for the patients’ clinical characteristics and several biochemical markers of outcomes in HF including plasma sodium level, eGFR, and hsCRP. But when BNP, one of the most powerful biomarkers in patients with HF, was included in the model, the prognostic performance of FeOxI activity was strongly reduced and did not become significant (P=0.053). From our results, we cannot argue whether high BNP and low FeOxI are only reflecting the severity of HF clinical situation of patients or whether they are directly pathogenetically linked.

In our study, we were unable to observe, as recently reported in the literature, a strong independent prognostic value of high ceruloplasmin circulating levels in stable patients undergoing elective coronary angiography and in a group of patients without HF or cardiovascular disease taken from the Atherosclerosis Risk in Communities Study.\textsuperscript{8,9} As a matter of fact, our findings show that ceruloplasmin and FeOxI were unrelated in patients with HF with pooled preserved and reduced EF. It is unclear why ceruloplasmin had no predictive value in our patients with HF; the majority (81%) of whom had an ischemic origin. However, the population enrolled in our study was significantly older (mean age, 76 years old), included patients with advanced stages of heart failure (~60% with mean EF of 34%) compared with the younger patients in the 2 larger studies by Tang et al\textsuperscript{8} and Dadu et al\textsuperscript{9} (mean age, 62 years old) and at least in the Atherosclerosis Risk in Communities Study in subjects without heart failure and cardiovascular disease at enrollment.\textsuperscript{9} Only FeOxI was found to predict the mortality risk, and a close inverse relationship was found with nitrotyrosine-bound ceruloplasmin in both patients with HF with preserved and reduced EF and with hsCRP in patients with HF with reduced EF. In addition, the analysis of hospitalization rate of our patient population showed that time to first admission was much shorter in patients with low FeOxI activity, whereas no difference in number of hospital admission during the follow-up period was observed among FeOxI tertiles. It should be noted that the number of hospitalizations recorded in our population of patients with HF is small, and there was insufficient power to detect a difference among tertiles.

Overall, our findings imply that a reduced antioxidative capacity as evidenced by decreased ceruloplasmin-related FeOxI activity plays a role in increasing the mortality risk in elderly patients with moderate-to-severe HF. Low FeOxI activity can in part affect the progression of left ventricular dysfunction as found in our study, but other mechanisms are involved and are probably more important. In fact, our data show that left ventricular EF was similar in the 2 tertiles with low FeOxI activity, thus suggesting the possibility that FeOxI activity may be more closely related to peripheral derangements and vascular alterations. In this regard, unfortunately, we did not measure vascular parameters in our patients, such as endothelial function, peak reactive hyperemia, or aerobic capacity, which could clearly influence the prognosis of patients with HF.
Our study has some other limitations. First, the results obtained from a single center require validation in large external cohorts of patients. Second, the clinical criteria of enrollment were restrictive: in fact patients with diabetes mellitus, a large portion in elderly HF population, were excluded. However, although the sample size is limited, a large number of deaths occurred during the follow-up period in the elderly patients with HF in our study, which made it possible to perform an analysis of FeOxI as an independent mortality predictor.

In conclusion, our findings provide evidence that in elderly patients with chronic HF, lower FeOxI activity related to higher ceruloplasmin nitration is associated with increased mortality risk. Both ceruloplasmin tyrosine nitration and cysteine thiol oxidation that are responsible in the in vitro setting of peroxynitrite-induced FeOxI activity inhibition may be the mechanisms also operant in vivo in patients with HF. However, further studies are required to deeper scrutinize these experimental and clinical research areas related to the effects of peroxynitrite on cysteine and tyrosine residues in ceruloplasmin, to evaluate the clinical significance of these results, and to establish whether preservation or restoration of FeOxI activity in HF could become a target for future treatment.

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Disclosures
None.

References


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**Novelty and Significance**

**What Is Known?**

- By regulating the balance between the toxic ferrous and the nontoxic ferric form of iron, serum ferroxidase protects against oxygen and nitrogen free radical injury.
- In human plasma, serum ceruloplasmin, the major copper transporter, has antioxidant properties via its ferroxidase I (FeOxI) activity.
- Advanced heart failure (HF) is associated with increased generation of aberrant oxygen free radicals and nitric oxide–derived peroxynitrite.

**What New Information Does This Article Contribute?**

- Low-serum FeOxI activity is associated with increased severity of heart failure and has important prognostic implications.
- Increased tyrosine nitration of ceruloplasmin is closely related to the drop in FeOxI activity in patients with HF.
- Cysteine thiol oxidation and tyrosine nitration of serum ceruloplasmin may be operant in vivo in peroxynitrite-induced FeOxI activity inhibition in patients with HF.

The FeOxI and ferroxidase II activities of serum ferroxidase regulate the balance between the toxic ferrous iron and nontoxic ferric form of iron and protect against iron-mediated oxygen and nitrogen-derived free radical injury. Ceruloplasmin, an α2-glycoprotein that transports serum copper, has antioxidant properties through its FeOxI activity. Increased peroxynitrite generation, as observed in HF, can affect protein folding and influence protein function through tyrosine nitration and cysteine sulfhydryl oxidation. However, in the HF population, the relationship between ceruloplasmin, peroxynitrite generation, tyrosine nitration, and FeOxI activity has not been established, and the clinical relevance of ceruloplasmin-mediated FeOxI activity remains unclear. We found that increasing severity of HF in patients, associated with a drop in serum FeOxI activity, is inversely related to ceruloplasmin tyrosine nitration. In experimental studies, both cysteine thiol oxidation and tyrosine nitration of serum ceruloplasmin were identified to be responsible for ex vivo and in vitro decrease in FeOxI activity. Thus, low FeOxI activity seems to have important prognostic implications in patients with chronic HF, and it may be an important target in HF treatment.
Low Serum Ferroxidase I Activity Is Associated With Mortality in Heart Failure and Related to Both Peroxynitrite-Induced Cysteine Oxidation and Tyrosine Nitration of Ceruloplasmin

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SUPPLEMENTAL MATERIAL

Low serum ferroxidase I activity is associated with mortality in heart failure and related to both peroxynitrite-induced cysteine oxidation and tyrosine nitration of ceruloplasmin.

Cabassi et al Ferroxidase I activity and heart failure

Methods

Cp Immunoprecipitation

For immunoprecipitation we used a polyclonal anti-human anti-Cp antibody (ABIN680108 antibodies-online GmbH, Aachen, Germany) that was cross-linked to Dynabeads protein A (Dynal Biotech, Oslo, Norway). Lyophilized rabbit anti-Cp antibody were reconstituted in purified distilled water to obtain a stock solution of 1ug/ul. After washing, 50 ul of Dynabeads (1.5 mg) were resuspended after magnetic separation (Dynal MPC) in 0.1 M Na₂HPO₄ pH 8.0, and transferred to a polypropylene test tube. The solution was incubated with rotation (Dynal MX1-Mixer) for 20 minutes at room temperature with 200 μl of phosphate buffer saline (pH 7.4) containing 2.5 μg of antibody. The supernatant was then magnetically removed and the beads-Ab complex resuspended with 200 μl phosphate buffer saline (pH 7.4) with 0.02% Tween 20 and washed by gently pipetting. Two hundred and fifty μl of diluted serum from the patients (1:20 with 0.1 M Na₂HPO₄ pH 8.0) were incubate with tilting and rotation for 45 minutes at room temperature. Test tubes were then placed on the magnet for 3 min to collect the beads beads-Ab complex on the tube wall. After washing 3 times (1 ml phosphate buffer saline, pH 7.4), immune complexes were released after incubation by tilting and rotation for 10 min at 25°C with 0.1 M glycine-HCl (pH 2.7) buffer. The tube was then placed on the magnet and the supernatant containing eluted antibody and Cp transferred to a clean tube. Eluted Cp solution was adjusted by adding 1 M Tris (Sigma T1503), to obtain a pH of 7.4. The protein concentration of the supernatant was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, California, USA). Appropriate control tests were established (one out of ten serum patient samples) by adding Cp to the serum at a known concentration and verifying its recovery. The eluted Cp was then tested for
nitrotyrosine with immunoblotting and enzyme-linked immunosorbent assays (ELISA assay Kit, Oxis Research International Inc. Foster City, CA USA).

**Immunoblotting for nitrotyrosine and cysteine thiol oxidation detection**

Eluates from Cp immunoprecipitation were subjected to standard SDS-PAGE and Western blotting techniques using 5% for stacking and 8-12% polyacrylamide separating gel and transferred to polyvinylidene difluoride membranes (Immun-Blot PVDF Membrane, Bio-Rad Laboratories, Hercules, California, USA) at 4 mA/cm² for 45 min as previously described (1). In brief, equal amounts of protein concentrations from each sample were loaded into the gel and run for 45 min at 200 V. A 12-μl volume of Rainbow molecular marker standards was used for molecular weight markers. Gel running buffer consisted of 1× Tris-glycine-SDS buffer. The membrane was then incubated with polyclonal anti-nitrotyrosine (1:2500; 06-284, Upstate, Millipore), and the signal amplified with an Opti-4CN Detection Kit (goat anti-rabbit HRP-conjugated secondary antibody, 1:10,000 Bio-Rad Laboratories, Hercules, California, USA). Nitrotyrosine-bound Cp was evaluated in serum from HF patients and CTR but also in *ex vivo* experiments on CTR serum (n=18) after incubation of ONOO⁻ or decomposed ONOO⁻ and in *in vitro* experiments (n=10) after ONOO⁻ or decomposed ONOO⁻ incubation of isolated purified Cp in a phosphate buffer bath.

In addition to nitrotyrosine, the evaluation of cysteine thiols oxidation after ONOO⁻ or decomposed ONOO⁻ incubation was performed on isolated purified Cp in a phosphate buffer bath. To detect cysteine thiol status we labeled untreated and ONOO⁻ or decomposed ONOO⁻-treated Cp free thiol groups with iodoacetamidofluorescein (IAF). Lyophilized Cp (50 ug/4,5 units, Sigma-Aldrich Chemical Co. St Louis, Missouri, USA) was dissolved in 350 μl of 0.2 M Tris-HCl (pH 7.40) and incubated for 10 min at 37°C in the presence or absence of ONOO⁻ and decomposed ONOO⁻ at a dose of 0.15 mmol/L. Samples were incubated with 0.5% SDS for 15 min at room temperature and then with 500 μM iodoacetamidofluorescein for 30 min in the dark at room temperature. Cysteine (5 mM) was added to quench any nonreacted iodoacetamidofluorescein. IAF-labeled thiol groups were detected using 1 mg/ml mouse anti-fluorescein-Oregon Green (Molecular Probes) diluted 1:4.000 into 1× Tris Buffer Saline with 0.05% Tween 20. Membranes were incubated with the appropriate primary antibody for 1 h at room temperature and then washed five times in 1× Tris Buffer Saline with 0.05%
Tween 20. Membranes were incubated again for 1 h with goat anti-mouse HRP-conjugated secondary antibody (31430, Pierce, ThermoFisher Scientific, Rockford, Illinois, USA) diluted 1:25.000 into 1× TBS with 0.05% Tween 20 and washed five times, for 5 min each, in fresh 1× TBS with 0.05% Tween 20 as previously described (2).

The result of the immunoblotting assay for IAF labeling of cystein thiol of isolated and lyophilized Cp dissolved in phosphate buffer before and after ONOO- incubation in presence and absence of GSH, EPI is depicted in Online Figure I.

**Online Figure I**

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<tr>
<td>ONOO\textsuperscript{-}, 150 μM</td>
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<td>EPI, 50 μM</td>
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Online Figure I: Representative immunoblotting assay for IAF labeling of cysteine thiol of isolated and lyophilized Cp before and after ONOO- incubation in presence and absence of GSH, EPI. Peroxynitrite (ONOO\textsuperscript{-})-induced decrease in iodoacetamidofluorescein labeling implies that peroxynitrite modifies cysteine thiol groups. Purified and lyophilized Cp was treated with 0.15 mM of ONOO- (lane 1 alone, lane 3 in presence of GSH, lane 5 in presence of EPI, lane 7 in presence of DMSO) or with decomposed (Dec. ONOO- (lane 2 alone, lane 4 in presence of GSH) or not treated (lane 6).
References
