Clinical and Genetic Association of Serum Ceruloplasmin With Cardiovascular Risk


Objective—Ceruloplasmin (Cp) is an acute-phase reactant that is increased in inflammatory diseases and in acute coronary syndromes. Cp has recently been shown to possess nitric oxide (NO) oxidase catalytic activity, but its impact on long-term cardiovascular outcomes in stable cardiac patients has not been explored.

Methods and Results—We examined serum Cp levels and their relationship with incident major adverse cardiovascular events (MACE; death, myocardial infarction [MI], stroke) over 3-year follow-up in 4177 patients undergoing elective coronary angiography. We also carried out a genome-wide association study to identify the genetic determinants of serum Cp levels and evaluate their relationship to prevalent and incident cardiovascular risk. In our cohort (age 63±11 years, 66% male, 32% history of MI, 31% diabetes mellitus), mean Cp level was 24±6 mg/dL. Serum Cp level was associated with greater risk of MI at 3 years (hazard ratio [quartile 4 versus 1] 2.35, 95% confidence interval [CI] 1.79–3.09, P<0.001). After adjustment for traditional risk factors, high-sensitivity C-reactive protein, and creatinine clearance, Cp remained independently predictive of MACE (hazard ratio 1.55, 95% CI 1.10–2.17, P=0.012). A 2-stage genome-wide association study identified a locus on chromosome 3 over the CP gene that was significantly associated with Cp levels (lead single-nucleotide polymorphism rs13072552; P=1.90×10−11). However, this variant, which leads to modestly increased serum Cp levels (∼1.5–2 mg/dL per minor allele copy), was not associated with coronary artery disease or future risk of MACE.

Conclusion—In stable cardiac patients, serum Cp provides independent risk prediction of long-term adverse cardiac events. Genetic variants at the CP locus that modestly affect serum Cp levels are not associated with prevalent or incident risk of coronary artery disease in this study population. (Arterioscler Thromb Vasc Biol. 2012;32:516-522.)

Key Words: coronary artery disease • nitric oxide • ceruloplasmin

Ceruloplasmin (Cp) is a circulating ferroxidase enzyme able to oxidize ferrous ions to less toxic ferric forms.1 It is the major carrier of circulating copper and is synthesized and secreted by the liver. Cp not only acts as a mediator of iron oxidation but also is an acute phase reactant in the setting of inflammation (such as infections or inflammatory arthritis). In contrast, inherited liver disorders such as Wilson disease may present with lower than normal levels of circulating Cp.

Recent studies support a role of Cp in regulating nitric oxide (NO) homeostasis.2 Isolated Cp was shown capable of catalytically consuming NO through NO oxidase activity, and plasma NO oxidase activity was decreased after Cp immu-nodepletion, in Cp knockout mice, and in people with congenital aceruloplasminemia.2 A mechanistic role for Cp in vascular disease beyond its association as an acute phase protein is thus suggested. Interestingly, myocardial uptake of Cp has been demonstrated in animal models,3 and epidemiological studies have linked Cp levels with cardiovascular risk both in apparently healthy individuals4–7 and in the setting of acute coronary syndromes.8–10 Despite the relative ease and affordability of Cp testing, few studies have examined Cp and its association with cardiovascular outcomes. The clinical prognostic value of Cp levels is not well understood, particularly in the contemporary era with statin therapy.

Methods

Study Population

The Cleveland Clinic GeneBank study is a large, prospective cohort study, run from 2001 to 2006, that established a well-characterized clinical repository with clinical and longitudinal outcomes data...
composed of consenting subjects undergoing elective diagnostic cardiac catheterization procedure. All GeneBank participants gave written informed consent approved by the Cleveland Clinic Institutional Review Board. This analysis included 4177 consecutive subjects of white European ancestry without evidence of myocardial infarction (MI; cardiac troponin I < 0.03 ng/mL) with plasma samples available for analysis. An estimate of creatinine clearance was calculated using the Cockcroft-Gault equation. The presence of coronary artery disease (CAD) was confirmed by luminal stenosis of at least 50% in any major coronary arteries. Major adverse cardiovascular event (MACE) was defined as death, nonfatal MI, or nonfatal cerebrovascular accident following enrollment. Adjudicated outcomes were ascertained over the ensuing 3 years for all subjects following enrollment.

**Cp Assay**

Quantitative determination of Cp was performed using an immuno-turbidimetric assay (Architect ci8200, Abbott, Abbott Park IL), which provides highly sensitive measurement of Cp levels with an intraassay coefficient of variation of 3.7%, interassay precision of up to 4%, and a reference range of 20 to 60 mg/dL. High-sensitivity C-reactive protein (hsCRP), myeloperoxidase (MPO), uric acid, creatinine, and fasting blood glucose and lipid profiles were measured on the same platform, as previously described.11

**Genotyping**

Genome-wide genotyping of single-nucleotide polymorphisms (SNPs) was performed on the Affymetrix Genome-Wide Human Array 6.0 chip in a subset of European ancestry patients in GeneBank. Using these data and those from 120 phased chromosomes from the HapMap CEU samples (HapMap r22 release, National Center for Biotechnology Information build 36), genotypes were imputed for untyped SNPs across the genome using MACH 1.0 software. All imputations were done on the forward (+) strand using MACH 1.0 software. All imputations were done on the forward (+) strand using MACH 1.0 software. All imputations were done on the forward (+) strand using MACH 1.0 software. All imputations were done on the forward (+) strand using MACH 1.0 software. All imputations were done on the forward (+) strand using MACH 1.0 software. All imputations were done on the forward (+) strand using MACH 1.0 software.

**Statistical Analyses**

The Student t test or Wilcoxon-Rank sum test for continuous variables and x² test for categorical variables were used to examine the difference between the groups. Kaplan-Meier analysis with Cox proportional hazards regression was used for time-to-event analysis to determine hazard ratio and 95% confidence intervals (95% CI) for 1-year and 3-year MACE. Levels of Cp were then adjusted for traditional cardiac risk factors in a multivariable model, including age, gender, diabetes mellitus, systolic blood pressure, low- and high-density lipoprotein, and triglyceride, as well as log-transformed hsCRP and creatinine clearance. Receiver operating characteristic curve analyses and 5-fold cross validation were used to determine the optimal cutoff. The improvement in model performance introduced by the inclusion of Cp was evaluated using net reclassification improvement index. The C statistic was calculated using the area under the receiver operating characteristic curve. To determine the optimal cutoff for Cp, we used a logistic regression model to estimate the risk of MACE. 5-fold cross validation divides the data into 5 approximately equally sized portions, and a logistic regression model is trained on 4 parts of the data and then estimates the risk of MACE in the fifth part. This is repeated for each of the 5 parts, and the area under the curve with the estimated risk was calculated. This process is carried out for a grid of values of Cp cutoff values, ranging from 17.3 mg/dL (10th percentile) to 31.5 mg/dL (90th percentile) with an increment of 0.1 mg/dL. The optimal cutoff is chosen to maximize area under the curve values. Probability values < 0.05 are considered statistically significant.

A genome-wide association study (GWAS) for serum Cp levels was carried out with adjustment for age and gender under an additive model. Linear regression analyses were carried out with PLINK (version 1.07) using log-transformed values. Unconditional multiple logistic regression was used to independently test for association of genetic variants with the presence and severity of CAD, with adjustment for age, gender, medication use (aspirin or statins), and Framingham Adult Treatment Panel III risk score (which includes smoking and diabetes status). Relative risk for experiencing a MACE was assessed using Cox proportional hazard models with adjustment for age, gender, medication use, and Framingham Adult Treatment Panel III risk score. These analyses were carried out assuming additive and dominant models. Adjusted odds or hazard ratios (OR or hazard ratio) with 95% CIs are reported with 2-sided probability values. All statistical analyses were performed using R version 9.2 (R Foundation for Statistical Computing, Vienna, Austria) and SAS version 9.2 (SAS Institute Inc, Cary, NC).

**Results**

Baseline characteristics of the study population are shown in Table 1. The mean and median serum Cp levels were 24 and 23 mg/dL (interquartile range 20–27 mg/dL), respectively. Patients with elevated Cp were more likely to be older and female, with more cardiovascular risk factors, including dyslipidemia, history of diabetes mellitus, and poorer renal function at baseline. There was strong direct correlation between Cp and hsCRP (r = 0.52, P < 0.001) but far weaker correlations with total leukocyte count (r = 0.15, P < 0.001) or...
MPO \( (r=0.12, P<0.001) \) and no correlation with serum uric acid levels \( (r=0.02, P=0.09) \).

### Association of Serum Cp Levels With Future MACE

Table 2 demonstrates the relationship between Cp levels in quartiles with 3-year risk for incident MACE. This graded risk was clearly illustrated in the Kaplan-Meier analysis (Figure 1A) when stratified according to quartile Cp ranges (quartiles 1 versus 4, hazard ratio: 2.35, 95% CI 1.79–3.09, \( P<0.001 \)). After adjustment for traditional risk factors, increased Cp levels remained significantly associated with incident major long-term major adverse cardiac events at 3 years (Table 2). The results were similar when stratified by gender, even though the cutoffs of the quartiles were higher in women (adjusted hazard ratio 1.77, 95% CI 1.13–2.77, \( P=0.013 \); fourth quartile Cp >31.5 mg/dL) than in men (adjusted hazard ratio 2.55, 95% CI 1.82–3.57, \( P<0.001 \); fourth quartile Cp >24.6 mg/dL). In particular, those with elevated Cp (cutoff at 22 mg/dL) and MPO (cutoff at 322 pg/mL), another known oxidase that contributes to catalytic consumption of NO within the vascular compartment,\(^{12–14}\) experienced the highest risk of developing future MACE (Figure 1B). Such prognostic value remained significant in various subgroups stratified by age, gender, presence of diabetes mellitus, hypertension, or renal insufficiency (Figure 2). When results were stratified by hsCRP or with MPO, we observed synergistic prediction of future MACE (Figure 3 and Table 2). Inclusion of Cp resulted in a significant improvement in risk estimation, based on net reclassification improvement index (9.6%, \( P<0.001 \)) and C statistic (67.7% versus 65.2%, \( P=0.003 \)).

### GWAS for Serum Cp Levels

We next performed a 2-stage GWAS for serum Cp levels in 4697 GeneBank subjects (all of European ancestry). In stage 1 \( (n=2647) \), the genomic inflation factor was 1.001, indicating that the GWAS results are not affected by underlying population (Figure 4). Serum Cp levels were primarily controlled by a single locus on chromosome 3, which con-
contains the CP gene itself. As shown in Table 3, the lead SNP at this locus (rs13072552) modestly but significantly increases of serum Cp levels by 2 to 3 mg/dL as a function of carrying 1 or 2 copies of the T allele (\( P = 3.63 \times 10^{-13} \)). The rs13072552 SNP has a minor allele frequency of 0.08, is located in intron 9 of CP, and is in strong linkage disequilibrium (\( r^2 = 0.7 \)) with the 2 other SNPs at this locus (rs11714000 and rs11921705) that exhibit similar association with Cp levels (data not shown). In stage 2, we genotyped 2050 additional GeneBank subjects in whom Cp levels were available and confirmed the association of rs13072552 (\( P = 2.42 \times 10^{-0.39} \)) in 4697 subjects used for the quantitative analyses described above, we genotyped rs13072552 in 3448 additional GeneBank subjects with available CAD phenotype data for these analyses (total \( n = 8145 \)). Under an additive model, the T allele of rs13072552, which increases serum Cp levels, was not associated with the presence or severity of CAD (Table 4). We also did not observe an association with history of MI in subjects with CAD or with future risk of MACE (Table 4) or when males and females were analyzed separately for MACE (\( P = 0.39 \) for males; \( P = 0.51 \) for females). Given the small number of subjects and to increase power, we also carried out these analyses using a dominant model with TT homozygotes grouped together with GT heterozygotes. However, these analyses also did not reveal an association with CAD, history of MI, or MACE (Table 4).

**Discussion**

A key finding of this study is the strong independent prognostic value of circulating Cp levels in stable patients undergoing cardiac evaluation in the contemporary statin era, above and beyond traditional cardiac risk factors, as well as cardiac and inflammatory risk markers. However, the effect of underlying genetic determinants of serum Cp levels were modest, and there was no gender-specific difference in prognostic value despite higher serum levels of Cp observed in women versus in men. With the broad availability and economic testing for serum Cp measurements in the clinical setting, these findings highlight the need to gain further insights into underlying pathophysiologic process.

**Association of CP Variants With Prevalent and Incident Risk of CAD**

We next sought to determine whether the genetic factors controlling Cp levels were associated with prevalent and incident risk of CAD. In addition to the 4697 subjects used for the quantitative analyses described above, we genotyped rs13072552 in 3448 additional GeneBank subjects with available CAD phenotype data for these analyses (total \( n = 8145 \)). Under an additive model, the T allele of rs13072552, which increases serum Cp levels, was not associated with the presence or severity of CAD (Table 4). We also did not observe an association with history of MI in subjects with CAD or with future risk of MACE (Table 4) or when males and females were analyzed separately for MACE (\( P = 0.39 \) for males; \( P = 0.51 \) for females). Given the small number of subjects and to increase power, we also carried out these analyses using a dominant model with TT homozygotes grouped together with GT heterozygotes. However, these analyses also did not reveal an association with CAD, history of MI, or MACE (Table 4).
related to increased serum Cp levels, which can affect long-term outcomes.

The precise role that Cp plays in the pathogenesis of cardiovascular morbidity and mortality has not been well described, although there has been extensive literature dating back to the 1970s describing the association between Cp and the heart, as well as its important oxidase activities. Patients with MI have higher observed serum Cp levels, which returned to normal ranges over time, suggesting its restrictive role as an acute phase reactant. Nevertheless, recent mechanistic studies show that Cp functions as an NO oxidase in vivo, suggesting that Cp elevations may lead to decreased NO bioavailability and endovascular dysfunction. Our findings are consistent with findings from prior epidemiological data in patients without known CAD. In the Helsinki Heart Study of dyslipidemic, middle-age men, higher serum Cp level (but not ferritin) was associated with graded increase in risk of cardiovascular events in a case-control comparison. Another prospective cohort study of elderly subjects also demonstrated a relationship between serum Cp levels and subsequent development of MI, whereas adjustment for hsCRP and leukocyte count reduced the excess risk by 33%. Unlike our present study population, the majority of patients in prior reports did not have prevalent cardiac diseases and were not treated with cardiac or lipid-lowering medications. It is interesting to observe that in our patient population with contemporary cardiac care, we still observed strong correlations between Cp and hsCRP levels but far weaker associations with MPO and leukocyte counts. Cytokines released from jeopardized tissues may stimulate the liver to synthesize acute phase proteins. Cp is one of these well-known inflammation-sensitive plasma proteins found to have protective effects in isolated rat hearts subjected to ischemia-reperfusion. The exact mechanisms for the cardioprotection are unclear but likely are due to a wide variety of extracellular antioxidative ferroxidase and reactive oxygen species scavenging effects, combined with putative glutathione-peroxidase and NO-oxidase/S-nitrosating activities. Whether such potentially beneficial effects are modified by oxidative stress and progress into vasculopathic factors that promote disease progression has been debated.

Figure 3. Event rates for major adverse cardiac events (MACE) stratified according to tertiles of ceruloplasmin (Cp), high-sensitivity C-reactive protein (hsCRP) (top), and myeloperoxidase (MPO, bottom).

Figure 4. Quantile-Quantile and Manhattan plots from genome-wide association study (GWAS) for serum ceruloplasmin levels. The probability values obtained from single-nucleotide polymorphisms (SNPs) in the GWAS analyses deviate from that expected by chance, suggesting that a subset of these signals indicate true associations (A). Serum ceruloplasmin levels in this study population (n=2647) are controlled predominantly by a locus on chromosome 3 containing the CP gene (B).
In the present study, we also used a GWAS approach to investigate the genetic determinants of serum Cp levels, which, to our knowledge, has not been previously reported. These analyses revealed that common genetic determinants linked to serum Cp levels in this white patient population are predominantly located within the CP locus. The 3 most significantly associated SNPs are all intronic and in strong linkage disequilibrium with each other. In addition, previous studies have shown that plasma concentrations of Cp are regulated at the posttranscriptional level. Specifically, a cis-regulatory element in the 3′ untranslated region of the CP transcript called GAIT (interferon-γ-activated inhibitor of translation) leads to selective translational silencing in myeloid cells.25,26

Despite the modest cis effect of the CP locus, our evaluation of the lead SNP (rs13072552) did not reveal an association with either the risk of CAD or future MACE. The lack of such an association could be due to several factors. For example, rs13072552 explains less than 1% of the variation in serum Cp levels, decreasing the power for detecting an association with cardiovascular outcomes. In addition, serum lipid and inflammatory markers, other genetic factors, and dietary/behavioral factors also contribute to the complex pathophysiology of CAD, MI, and MACE. The subjects used in our study were also recruited from patients undergoing elective cardiac catheterization, which results in a study population skewed toward individuals mostly having documented CAD and being treated with medications to lower their risk of future MACE. Such confounders, taken together with the low minor allele frequency of rs13072552 and its modest effects on serum Cp levels, in combination with the recognition that posttranscriptional processes play a major role in Cp production, may explain, in part, the negative association of the CP locus with CAD and MACE. Our study is also limited by only including subjects of white European ancestry, and additional studies in other ethnicities and with increased power will be required to determine whether the negative genetic findings hold true. However, it is interesting to note that our observations with Cp are similar to other inflammation-sensitive plasma proteins, such as C-reactive protein, where consistent prognostic effects were not confirmed by genetic predisposition of cardiovascular risk in relatively large data sets.27 Thus, further studies are also warranted to explore the molecular mechanism underlying the strong association between serum Cp levels and cardiovascular events beyond those observed with alternative acute phase proteins (eg, hsCRP), leukocyte counts, and traditional cardiac risk factors.

The recent discovery of a potential role for Cp as a catalytic sink for NO consumption in vivo raises new and important questions about the vascular function(s) and clinical utility of this unusual copper-containing protein. The addition of Cp testing, a readily available and affordable assay, may provide synergistic prognostic value with traditional cardiac risk factors and alternative inflammation markers, such as hsCRP, MPO, and leukocyte count. In an era of cost effectiveness, additional studies are warranted to determine whether Cp testing may provide value in prioritizing preventive interventions among those with unrecognized heightened cardiovascular risks.

### Table 3. Association of rs13072552 With Serum Cp Levels

<table>
<thead>
<tr>
<th>Stage</th>
<th>MAF</th>
<th>GG</th>
<th>GT</th>
<th>TT</th>
<th>P Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>GWAS (n=2647)</td>
<td>0.081</td>
<td>23±6 (n=2234)</td>
<td>25±6 (n=393)</td>
<td>28±8 (n=20)</td>
<td>3.63×10⁻¹³</td>
</tr>
<tr>
<td>Replication (n=2050)</td>
<td>0.077</td>
<td>25±6 (n=1774)</td>
<td>26±6 (n=263)</td>
<td>24±9 (n=13)</td>
<td>2.42×10⁻²</td>
</tr>
<tr>
<td>Combined (n=4697)</td>
<td>0.079</td>
<td>24±6 (n=4008)</td>
<td>25±6 (n=656)</td>
<td>26±9 (n=33)</td>
<td>1.90×10⁻¹¹</td>
</tr>
</tbody>
</table>

Mean serum Cp levels are shown as a function of genotype for rs13072552. MAF indicates minor allele frequency.

P values were obtained using log-transformed Cp values and after adjustment for age and gender.

### Table 4. Association of rs13072552 With Prevalent and Incident Risk of CAD

<table>
<thead>
<tr>
<th>Trait</th>
<th>GG (n=6941)</th>
<th>GT (n=1130)</th>
<th>TT (n=74)</th>
<th>P Value</th>
<th>GT/TT (n=1204)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No CAD vs CAD⁺, OR (95% CI)</td>
<td>0.99 (0.84–1.17)</td>
<td>1.44 (0.72–2.87)</td>
<td>0.74</td>
<td>1.01 (0.86–1.19)</td>
<td>0.92</td>
<td></td>
</tr>
<tr>
<td>No disease vs mild disease, OR (95% CI)*</td>
<td>1.02 (0.78–1.09)</td>
<td>0.61</td>
<td>0.94 (0.80–1.10)</td>
<td>0.44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No disease vs severe disease, OR (95% CI)*</td>
<td>1.04 (0.87–1.24)</td>
<td>0.69 (0.35–1.39)</td>
<td>0.93</td>
<td>1.02 (0.86–1.21)</td>
<td>0.84</td>
<td></td>
</tr>
<tr>
<td>CAD⁺/Mi⁻ vs CAD⁺/Mi⁺, OR (95% CI)</td>
<td>0.97 (0.83–1.13)</td>
<td>1.02 (0.60–1.74)</td>
<td>0.77</td>
<td>0.97 (0.84–1.13)</td>
<td>0.72</td>
<td></td>
</tr>
<tr>
<td>Future MACE, HR (95% CI)</td>
<td>1.08 (0.91–1.28)</td>
<td>1.32 (0.78–2.23)</td>
<td>0.22</td>
<td>1.09 (0.93–1.29)</td>
<td>0.28</td>
<td></td>
</tr>
</tbody>
</table>

ORs and HRs were calculated with adjustment for age, gender, medication use (aspirin and/or statins), and Framingham risk score. OR indicates odds ratio; HR, hazard ratio.

*Disease severity was defined as having ≥50% stenosis in 1 or 2 (mild) or ≥3 (severe) major epicardial arteries.
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Disclosures

Dr Tang received research grant support from Abbott Laboratories and served as a consultant for Medtronic Inc and St. Jude Medical.

Dr Hazen is named as coinventor on pending and issued patents held by the Cleveland Clinic relating to cardiovascular diagnostics. Dr Hazen has been paid as a consultant or speaker for the following companies: Cleveland Heart Lab, Inc.; Esperion; and Pfizer Inc. Dr Hazen has received research funds from Abbott; Cleveland Heart Lab, Inc.; Esperion; and Liposciences Inc.; Dr Hazen has the right to receive royalty payments for inventions or discoveries related to cardiovascular diagnostics from Abbott Laboratories; Cleveland Heart Lab, Inc.; Frantz Biomarkers, LLC; and Siemens.

References

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### Supplement Material

**Supplemental Table I. Association of rs13072552 with Serum Cp Levels Stratified by Gender.**

**Males**

<table>
<thead>
<tr>
<th>Stage</th>
<th>MAF</th>
<th>GG</th>
<th>GT</th>
<th>TT</th>
<th>*p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GWAS (n = 1880)</td>
<td>0.081</td>
<td>22 ± 5</td>
<td>23 ± 5</td>
<td>25 ± 7</td>
<td>2.25 x 10^-8</td>
</tr>
<tr>
<td>Replication (n = 1288)</td>
<td>0.075</td>
<td>23 ± 5</td>
<td>24 ± 5</td>
<td>23 ± 10</td>
<td>0.15</td>
</tr>
<tr>
<td>Combined (n = 3168)</td>
<td>0.079</td>
<td>22 ± 5</td>
<td>23 ± 5</td>
<td>24 ± 9</td>
<td>2.92 x 10^-07</td>
</tr>
</tbody>
</table>

**Females**

<table>
<thead>
<tr>
<th>Stage</th>
<th>MAF</th>
<th>GG</th>
<th>GT</th>
<th>TT</th>
<th>*p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GWAS (n = 767)</td>
<td>0.083</td>
<td>27 ± 6</td>
<td>30 ± 7</td>
<td>32 ± 9</td>
<td>1.72 x 10^-5</td>
</tr>
<tr>
<td>Replication (n = 762)</td>
<td>0.063</td>
<td>28 ± 6</td>
<td>29 ± 6</td>
<td>28 ± 4</td>
<td>4.84 x 10^-02</td>
</tr>
<tr>
<td>Combined (n = 1529)</td>
<td>0.073</td>
<td>28 ± 6</td>
<td>30 ± 7</td>
<td>31 ± 8</td>
<td>7.36 x 10^-06</td>
</tr>
</tbody>
</table>

Mean serum Cp levels as a function of genotype for rs13072552. Minor allele frequency (MAF).

*p-values obtained using log transformed Cp values and after adjustment for age.*