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Plasma Ceruloplasmin, A Regulator of Nitric Oxide Activity, and Incident Cardiovascular Risk in Patients With CKD

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Abstract

Background and objectives Increased serum levels of the acute-phase reactant ceruloplasmin predict adverse clinical outcomes in the setting of acute coronary syndromes and heart failure, but their role in patients with CKD is unclear. This study investigated the relationship of ceruloplasmin with clinical outcomes in CKD, especially with regard to traditional cardiac biomarkers.

Design, setting, participants, & measurements Serum ceruloplasmin levels in consecutive study participants with CKD (n=654; estimated GFR<60 ml/min per 1.73 m^2) as well as a control group of non-CKD participants matched for age and sex (n=250) were measured. Study participants were enrolled during 2001 2006 from a population of patients presenting for elective diagnostic coronary angiography and prospectively followed for 3 years (median follow-up=1095 days) to determine incident major adverse cardiac events (defined as a composite of death, nonfatal myocardial infarction, and stroke).

Results Serum ceruloplasmin levels in CKD patients were elevated versus controls (median [interquartile range]; 25.5 [21.8 29.6] versus 22.7 [19.7 26.5] mg/dl; P<0.001) and associated with increased risk of future major adverse cardiac events (hazard ratio, 1.35; 95% confidence interval, 1.0 to 1.82; P=0.04). After adjusting for traditional risk factors, higher serum ceruloplasmin was still associated with higher risk of major adverse cardiac events at 3 years (hazard ratio, 1.61; 95% confidence interval, 1.15 to 2.25; P=0.01).

Conclusion In CKD patients, increased serum ceruloplasmin, a regulator of nitric oxide activity, is associated with increased risk of long-term adverse cardiovascular events, even after multivariable model adjustment for traditional clinical and biologic risk factors.

Introduction

CKD is a nitric oxide (NO)-deficient state (1), and the associated oxidative stress contributes to not only the progression of renal injury, but also its attendant cardiovascular complications (2). NO imbalance can promote inflammation, oxidant stress, endothelial dysfunction, and tissue injury, and it is a potential contributor to both CKD and cardiovascular disease progression.

Ceruloplasmin (Cp) is an acute-phase reactant that is synthesized and secreted by the liver as well as monocyte/macrophages, and it participates in both iron and copper metabolism (3). Recently, both antioxidant and pro-oxidant activities of Cp have been shown and suggest a role for Cp in regulating NO homeostasis (4). *In vitro*, Cp shows NO oxidase activity by catalytic consumption of NO, whereas Cp immunodepletion, Cp knockout mice, and humans with congenital aceruloplasminemia all show diminished plasma NO oxidase activity (4). Several studies have also connected Cp levels with increased cardiovascular risk in both the normal population (5 7) and

patients with acute coronary syndromes (8,9). In particular, our group has shown the prognostic value of elevated Cp, even after adjustment for traditional risk factors, for determining the future risk of cardiac events in stable cardiac patients (10).

Despite significant evidence that enhanced oxidative and nitrative stresses are present in patients with CKD and ESRD, the underlying pathways involved in this imbalance remain a topic of active investigation. In this study, we sought to establish the possible role of serum Cp in predicting incident adverse clinical outcomes, including myocardial infarction (MI), stroke, and death, among patients with moderate CKD.

Materials and Methods
Study Population

We performed serum Cp measures in samples collected from a prospective cohort of 654 successive individuals with CKD (estimated GFR [eGFR]<60 ml/min per 1.73 m^2) presenting for elective diagnostic cardiac evaluations at our health system. The

study population was enrolled during 2001 2006; they were followed prospectively for 3 years (median follow-up=1095 days), and they consisted of only stable study participants undergoing elective diagnostic coronary angiography (either cardiac catheterization or coronary computed tomography angiography) not in the setting of acute coronary syndrome (cardiac troponin I<0.03 μg/L). No study participants were on dialysis. A control group of non-CKD participants (eGFR$\geq$60 ml/min per 1.73 m^2, n=250) was obtained from the same setting and matched for age and sex (1:2.6 versus the CKD group). Venous blood samples collected before any heparin administration were obtained in all study participants in serum separator tubes, processed, aliquoted, and stored at 80°C within 4 hours of collection. Written informed consent was obtained for all study participants. The Institutional Review Board of the Cleveland Clinic approved the study protocols, and the study adhered to the Declaration of Helsinki.

Study evaluation included assessment of standard cardiac risk factors, including age, sex, systolic BP, and fasting lipids, as well as history of diabetes mellitus, cigarette smoking, MI, or heart failure. eGFR was calculated according to the four-variable equation of the Kidney Disease Outcomes Quality Initiative Modification of Diet in Renal Disease study guidelines (11).

End Points

Definition of major adverse cardiovascular events (MACEs) included death, nonfatal MI, or nonfatal cerebrovascular accident. Study staff ascertained end points through in-person follow-up, which included written solicitation and reply cards, medical chart review, and direct contact. Adjudicated outcomes were collected for all study participants over the 3-year follow-up period.

Serum Biochemical Assays

Quantitative measurement of Cp was performed using an immunoturbidimetric assay (Abbott Architect ci8200; Abbott, Abbott Park, IL), which yields high-sensitivity measurement of Cp levels. The Cp turbidimetric procedure was calibrated at least every 10 days by using multi-calibrator material, which was traceable to Reference Preparation for Protein in Human Serum lot number 91/ 0619. During operation of the Abbott analyzer, at least two levels of control material were tested a minimum of one time per day. The intra-assay coefficient of variation was 3.7%, with an interassay precision of up to 4% over a reference range of 20 60 mg/dl. All other assays, including high-sensitivity C-reactive protein (CRP), creatinine, and fasting blood glucose and lipid profiles, were measured on the Abbott Architect platform as previously described (12).

Statistical Analyses

Continuous variables were compared using the t test (for normal distribution) or Wilcoxon rank sum test (for non-normal distribution), whereas the chi-squared test was used for categorical variables. The relationship between serum Cp levels and other biologic measures was determined by the Spearman correlation. The log-linearity assumption of Cox models was assessed by introduction of a cubic spline component for each continuous variable.

Receiver operator characteristic curve analyses in the context of the time to event were performed to determine the optimal cutoff at <25.5 mg/dl Cp level (which was also the median Cp level), with a 5-fold cross-validation Cox model used to estimate risk of event. Using the 5-fold crossvalidation methods, the data are divided into five approximately equally sized portions; the Cox model is trained on four parts of the data, and then, it estimates the risk of MACE in the fifth part. This process is repeated for each of the five parts, and area under the curve with the estimated risk is then calculated. This iterative process was carried out for Cp cutoff values ranging from 19.5 to 34.5 with an increment of 0.1, and the optimal cutoff was chosen to maximize the C statistics. Quantification of model performance improvement was determined by both Net Reclassification Improvement (NRI) and Integrated Discrimination Improvement, and low-, medium-, and high-risk category cutoff values for NRI estimation used a ratio of 6:3:1, respectively. P values represent comparison of models with and without Cp. Models adjustment for established risk factors included age, sex, smoking, HDL cholesterol, systolic BP, history of diabetes mellitus, triglyceride, eGFR, serum albumin, and BUN. Survival curves of the two respective Cp groups (serum Cp<25.5 versus $\geq$25.5 mg/dl) were compared with Kaplan Meier analysis and the log-rank test. Time-to-event analysis used to determine hazard ratios (HRs) and 95% confidence intervals (95% CIs) for MACE was determined by Cox proportional hazards regression, and adjustment was made for established cardiac risk factors, including age, sex, systolic BP, cigarette smoking, fasting cholesterol values (including LDL and HDL cholesterol levels), and eGFR. Additional adjustments incorporated medication use (including angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, β-blockers, and statin therapy), serum albumin, BUN, history of MI, and history of heart failure to predict incident 3-year MACE risks. Statistical significance was defined as P<0.05 using SAS version 8.2 (Cary, NC) and R 2.8.0 (Vienna, Austria).

Results

Subject Characteristics

The baseline characteristics for the study population are presented in Table 1. For the CKD group, mean and median eGFR were 46$\pm$13 and 50 ml/min per 1.73 m^2 (interquartile range=40 56 ml/min per 1.73 m^2), respectively. Serum Cp in the CKD group was higher compared with serum Cp in the non-CKD control group (median [interquartile range]; 25.5 [21.8 29.6] versus 22.7 [19.7 26.5] mg/dl; P<0.001) (Figure 1). Within the CKD group, serum Cp was correlated with high-sensitivity CRP (r=0.43, P<0.001), but it did not correlate with indices of renal function, such as eGFR (r= 0.04, P=0.26) and BUN (r<0.001, P=0.96).

Serum Cp and Major Adverse Cardiac Outcomes

Within the 3-year follow-up period (median follow-up=1095 days), a total of 174 events (nonfatal MI, stroke, or death) was recorded. Dividing Cp as a dichotomous variable according to the cutoff of 25.5, higher serum Cp (>25.5 mg/dl) was associated with development of

Table 1. Baseline subject characteristics

Variable	eGFR 60 ml/min per 1.73 m^2 (n=250)	eGFR<60 ml/min per 1.73 m^2 (n=654)	PValue
Age (yr)	68:t9	70:tlO	0.10
Men(%)	53	54	0.86
Diabetes(%)	20	52	<0.001
Hypertension(%)	60	86	<0.001
Cigarette smoking(%)	59	61	0.63
Prior HF(%)	13	39	<0.001
Prior MI(%)	0	48	<0.001
I D L cholesterol (mg/dl)	100 (82 117)	92 (73 114)	<0.001
HDL cholesterol (mg/dl)	39 (33 45)	32 (26 40)	<0.001
Triglycerides (mg/dl)	98 (75 131)	133 (96 190)	<0.001
hsCRP (mg/L)	2 (1 4)	4 (2 9)	<0.001
Framingham Risk Score	10 (7 12)	11 (8 13)	<0.001
eGFR (ml/min per 1.73 m^2)	82 (74 92)	SO (40 56)	<0.001
Baseline medications			
ACE inhibitors/ARBs (%)	32	63	<0.001
/3-Blockers (%)	32	59	<0.001
Stalin(%)	38	66	<0.001
Aspirin(%)	58	66	0.02

Values expressed as mean:!:SD or median (interquartile range). eGPR, estimated GPR; HF, heart failure; MI, myocardial infarction; hsCRP, high sensitivity C reactive protein; **ACE,** angiotensin converting enzyme; **ARB,** angiotensin receptor blocker.
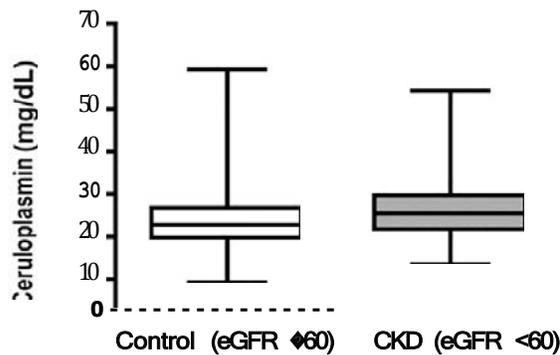

Figure 1. I Comparison of serum ceruloplasmin between control participants (estimated GFR [eGFR]i!:60 ml/min per 1.73 m^2) and participants with CKD (eGFR<60 ml/min per 1.73 m^2). P<0.001 by both Wilcoxon and ttests.

adverse cardiac events (HR, 1.35; 95% CI, 1.0 to 1.82; P=0.04) (Table 2). After adjustment for established risk factors, such as HDL, history of MI, history of heart failure, and medication use (including angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, /3-blockers, and statin therapy), elevated Cp was still associated with higher risk of MACEs at 3 years (HR, 1.69; 95% CI, 1.18 to 2.42; P=0.003). Performance of the risk analysis was according to quartiles of serum Cp, and it also showed that the highest serum Cp quartile (>29 6 mg/dl) was predictive of future development of MACEs at 3 years after adjusting for traditional risk factors and medication use (HR, 1.89; 95% CI, 1.17 to 3.07; P=0.01) (Supplemental Table 1). Similar significant trends were noted after additional adjustments for both serum albumin and BUN (Supplemental Table 1, Table 2), and they were also noted in the control group (Supplemental Tables 2 and 3). Moreover, the addition of serum Cp to traditional risk factors, including eGFR, resulted in significant integrated discrimination improvement (Integrated Discrimination lmprovement=6%; P<0.001) and significant event-specific net reclassification (paraoxonase NRl=ll.6%; P<0.001). Kaplan Meier survival analyses showed that elevated Cp levels within the CKD study participants were associated with higher event rates compared with lower Cp levels (log rank P=0.04) (Figure 2).

Discussion

Cp is a major hypoxia-activated systemic N O oxidase that seems to be able to function in both antioxidant and pro-oxidant capacities (13). In the current study, we provide evidence of a significant elevation in serum Cp in a large cohort of stable patients with moderate CKD undergoing elective coronary angiography compared with an age- and sex-matched control group without CKD. Furthermore, we show a significant association between high Cp and poor long-term prognosis independent of traditional cardiac risk factors. Although elevated Cp is associated with cardiovascular events in other populations of healthy individuals (5 7), the setting of acute coronary syndromes (8,9), and stable cardiac patients (10), this study is the first study, to our knowledge, that examines the relationship between serum Cp and clinical outcomes in patients with CKD. It is conceivable that future studies designed to modulate Cp's cardio- and possibly even renoprotective effects will present novel therapeutic strategies in this population, which suffers from significant cardiovascular morbidity and mortality.

There are several important questions raised by our findings. First, is the elevated Cp in the setting of CKD a

protective mechanism aimed at neutralizing uremic toxins in this high-risk group, or is it rather itself a toxic factor, which promotes or accelerates cardiovascular risk in this

population? Second, what mechanisms lead to Cp elevations in CKD, and what are the consequences of elevated Cp for these patients? Although our data suggest that, in the setting of CKD, elevated Cp is associated with worse prognostic outcome and that Cp is associated with inflammatory markers, such as CRP, whether or not elevated Cp is a positive or negative counter-regulatory biologic response will await additional mechanistic studies. To be sure, the fact that Cp added prognostic value to the Cox models even after adjustment for eGFR suggests that the generation of Cp is likely a metabolic response related to CKD and independent of glomerular filtration.

The precise pathophysiologic mechanism, where increased Cp may lead to MACEs in CKD, is not fully understood. Elevated Cp may be directly linked to heightened downstream nitrative stress in CKD. In the setting of renal disease, increased NO oxidase activity coupled with depressed endogenous NO synthase mechanisms create a redox imbalance that favors oxidative and nitrative stress. Cp can form reactive oxidant species through catalyzing NO oxidase activity in the circulation and promoting NO oxidation to form nitrogen dioxide (N) (4), a reactive nitrogen species involved in initiation of Cp-mediated lipid peroxidation and protein nitration (14,15). In this way, elevated Cp may promote atherosclerotic mechanisms that may underlie the cardiovascular events observed in our study. Furthermore, in the setting of ischernic-reperfusion injury animal models, plasma NO oxidase activity was decreased after Cp immunodepletion (4), suggesting that the elevated NO oxidase activity in CKD may be linked to Cp elevations. Elevated Cp is also involved in the impairment of the endothelium-dependent vasodilatation (16), which is another potential mechanism driving the accelerated cardiovascular events in this population.

Alternatively, increased Cp production may be simply a homesostatic mechanism for responding to the uremic toxins that accumulate in CKD. As an antioxidant, Cp

Table 2. Unadjusted and adjusted 3-year hazard ratio for major adverse cardiac events at 3 years stratified by optimal cutoff values for serum ceruloplasmin

Variable	Ceruloplasmin, mg/dl (range)	
	<25.5	25.5
3-YearMACE	78/327 (23.9%)	96/327 (29.4%)
Unadjusted HR	1	1.35 (1 1.82][a]
Adjusted HR: Model 1	1	1.59 (1.14 2.22[a]
Adjusted HR: Model 2	1	1.61 (1.15 2.25[a]
Adjusted HR: Model3	1	1.61 (1.14 2.25[a]
Adjusted HR: Model4	1	1.72 (1.2 247][b]
Adjusted HR: Models	1	1.69 (1.18 2.42[a]

Model 1: adjusted for traditional risk factors, including age, sex, systolic BP, LDL cholesterol, HDL cholesterol, smoking, and diabetes mellitus. Model 2: adjusted for traditional risk factors, angiotensin converting enzyme (ACE) inhibitor, angiotensin receptor blocker (ARB), β blocker, and statin use. Model 3: adjusted for traditional risk factors and BUN. Model 4: adjusted for traditional risk factors, history of heart failure (HF), and history of MI. Model 5: adjusted for traditional risk factors, history of HF, history of MI, ACE inhibitor, ARB, β blocker, statin use, and BUN. MACE, major adverse cardiac event; HR, hazard ratio.
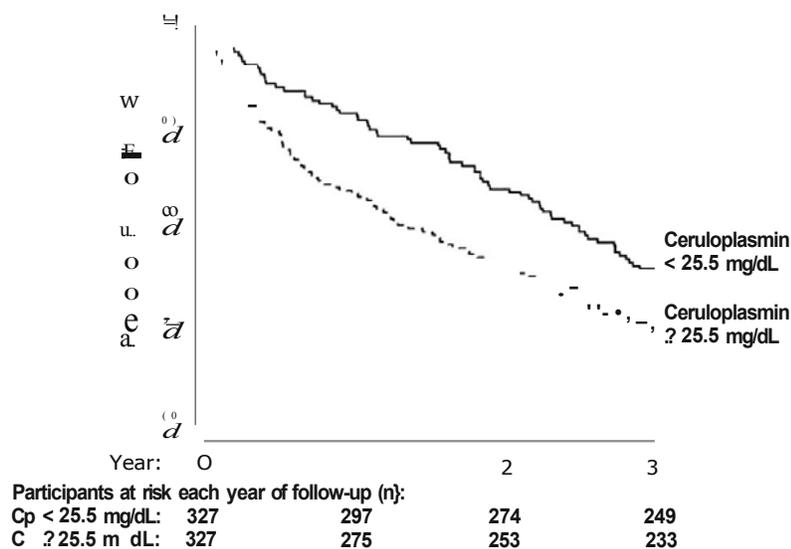[a]P<O.C6.
[b]P<0.01.

Figure 2. I Kaplan-Meier analysis of major adverse cardiac events (MACEs) in participants with CKO. Participants were stratified according to optimal cutoff for serum ceruloplasmin (Cp): low Cp (<25.5 mg/di; solid line) or high Cp (2:25.5 mg/di; dashed line).

serves as a ferroxidase that converts ferrous iron to a nontoxic ferric iron, thus allowing for its binding to, and transport by, transferrin. In this way, Cp serves to remove free ferrous iron and, thereby, reduces a major producer of superoxide and hydroxyl radical oxidants. Cp can also serve as a general antioxidant by both catalyzing the destruction of oxygen radicals as well as inhibiting the oxidant activity of myeloperoxidase (17). Therefore, there may be a homeostatic balance between pro-oxidant and antioxidant mechanisms that prevails under different conditions. In the setting of limited NO availability, the NO oxidase function of Cp might promote nitrative stress and disease progression, whereas in the setting of excessive oxidant production, the antioxidant effects of Cp may predominate.

The mechanisms leading to the Cp elevations that we observed in patients with CKD remain unclear. Tubulointerstitial and glomerular hypoxia are important factors that accelerate renal disease (18), and recent studies have observed that activation of Cp expression is mechanistically linked to a hypoxia-inducible factor, where hypoxia response element dependent gene regulation leads to transcriptional induction of the Cp gene promoter (19). These data, together with the intermittent but recurring hypoxia in the setting of CKD (20), suggest that extrinsic factors, such as hypoxia, may be responsible for modulating Cp levels in patients with CKD. Furthermore, in a rat model of age-associated glomerulosclerosis, caloric restriction prevented elevations in both secreted Cp and Bowman's capsule Cp expression and reduced glomerulosclerosis at 24 months (21). It was further shown that NF-κB p50 binds to the κB motif of Cp expressed in the parietal epithelium in old versus young glomeruli, suggesting an age-associated NF-κB driven process (22).

Although Cp has established links with measures of systemic oxidant/nitrative stress and cardiovascular risk in patients with normal renal function (5 10), we now report that serum Cp levels may be prognostically important within a large cohort of patients with moderate CKD and prospective long-term clinical event data. Our findings suggest that Cp may be a potentially important regulator of oxidative and nitrative stress that is increased in patients with moderately reduced renal function. Because Cp is readily measured in any clinical chemistry laboratory, it may be a clinically useful diagnostic and therapeutic target in the setting of CKD, because treatment options that address the significant burden of cardiovascular morbidity and mortality in these patients are limited. Our findings suggest that specific oxidative and nitrative stress pathways, such as those pathways catalyzed by Cp, may be an important modifier of cardiovascular risk in patients with moderate CKD, and thus, it is a relevant topic for future mechanistic investigation.

Study Limitations

The selection of stable stage 3 CKD patients with suspect coronary disease may introduce a selection bias that potentially confounds interpretation of such case-control studies. Serum Cp was also only measured at a single time point, and, as such, we have limited ability to detect the influence of therapeutic effects on Cp levels over time or whether changes in Cp levels over time are also of prognostic importance to patients with CKD. Similarly, because we do not have data, such as proteinuria/albuminuria or follow-up creatinine, on these patients, we are limited in our ability to fully characterize their underlying renal disease. Because we do not have complete Cp polymorphism data on these patients, we are also unable to assess the contribution of genetic factors in these patients. Despite these limitations, this study represents the first examination, to our knowledge, of the contribution of Cp to cardiovascular outcomes in a large cohort of well characterized patients with moderate CKD. Although such clinical associative studies do not allow a complete characterization of the etiology of biomarkers, such as Cp, they do provide an important framework on which additional mechanistic studies can build. Because patients with CKD bear a significant burden of oxidative and nitrative stress, as well as cardiovascular morbidity and mortality, our findings merit follow-up examination into this potentially novel oxidative and nitrative stress pathway in CKD.

Elevated Cp levels are associated with increased risk for development of adverse cardiac events, including nonfatal MI, nonfatal stroke, or death, in patients with CKD, even after multivariable models adjustment for established clinical and biologic risk factors. These findings are supportive of the hypothesis that oxidative and nitrative stresses play a significant role in mediating cardiac disease progression in CKD and point to the potential pro-oxidant role of Cp in this setting.

Disclosures

D.J.K., Y.F., Y.W., M.P., and W.H.W.T. report no relationships to disclose. S.L.H. is named as coinventor on pending patents held by the Cleveland Clinic relating to cardiovascular diagnostics. S.L.H. reports payment as a consultant or speaker for Cleveland Heart Laboratory, Esperion, Lilly, Liposcience, Inc., Merck & Co., Inc., and Pfizer, Inc., as well as receiving research funds from Abbott, Cleve land Heart Laboratory, Liposcience, Inc., and Pfizer, Inc. S.L.H. re ports having the right to receive royalty payments for inventions or discoveries related to cardiovascular diagnostics or therapeutics from the following companies: Abbott Laboratories, Inc., Cleveland Heart Laboratory, Esperion, Frantz Biomarkers, LLC, Liposcience, Inc., and Siemens.

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