Association of Catalytic Iron With Cardiovascular Disease

Mohan M. Rajapurkar, MD[a], Sudhir V. Shah, MD[c,d,*], Suhas S. Lele, MD[e], Umapati N. Hegde, MD, DNB[a], Shelly Y. Lensing, MS[c], Kalpesh Gohel, MD, DNB[a], Banibrata Mukhopadhyay, MSc, PhD[b], Sishir Gang, MD, DM, DNB[a], and Marsha L. Eigenbrodt, MD, MPH[c]

The ability of iron to cycle reversibly between its ferrous and ferric oxidation states is essential for the biological functions of iron but may contribute to vascular injury through the generation of powerful oxidant species. We examined the association between chemical forms of iron that can participate in redox cycling, often referred to as "catalytic" or "labile" iron, and cardiovascular disease (CVD). In our cross-sectional study of 496 participants, 85 had CVD. Serum catalytic iron was measured using the bleomycin-detectable iron assay that detects biologically active iron. The odds of existing CVD for subjects in the upper third of catalytic iron were 10 times that of subjects with lower catalytic iron in unadjusted analyses. The association was decreased by 1/2 by age adjustment, but little additional attenuation occurred after adjusting for age, Framingham Risk Score, estimated glomerular filtration rate, hypertension status, high-density lipoprotein cholesterol, and systolic blood pressure, with the association remaining strong and significant (odds ratio 3.8, 95% confidence interval 1.4 to 10.1). In conclusion, we provide preliminary evidence for a strong detrimental association between high serum catalytic iron and CVD even after adjusting for several co-morbid conditions; however, broader prospective studies are needed to confirm these findings, which would support therapeutic trials to assess the beneficial effects of iron chelators on CVD. Published by Elsevier Inc. (Am J Cardiol 2012;109:438–442)

Cardiomyopathy is known to occur in several iron overload states[1]; however, a role for iron in atherosclerotic cardiovascular disease (CVD) in the absence of iron overload is less clear.[2–5] Although several in vitro[6] and animal[7,8] studies have supported a role for iron in atherosclerosis, human observational studies have provided inconsistent results.[3–5,9–11] Several factors, including the fact that total body iron is not reliably related to the level of biologically active iron,[12,13] may have contributed to these inconsistencies.[13] In the present study we evaluated groups of patients with several long-term conditions to assess the association between serum catalytic iron and CVD after controlling for various co-morbidities.

Methods

This cross-sectional study was approved by the institutional ethics committees at the participating centers, and written informed consent was obtained from all study participants. The 568 subjects who agreed to participate included 349 participants from a survey of healthy governmental workers, 147 patients with chronic kidney disease (CKD) who attended Muljibhai Patel Urological Hospital, and 72 patients with angiographically established stable coronary artery disease who attended cardiology clinics at Bhaila Amin General Hospital. To adjust for the Framingham 10-year coronary heart disease (CHD) risk score,[14] subjects <20 years of age and those ≥ 80 years of age were excluded. Given the potential impact of hemodialysis on laboratory values, we excluded 58 patients on hemodialysis, leaving 496 participants for analyses.

All subjects underwent a detailed clinical evaluation from July 2007 through June 2008 at the Muljibhai Patel Urological Hospital, where risk factor profiles and medical histories including medical diagnoses and tobacco use were carefully recorded. Body mass index was calculated as weight (kilograms) divided by height (meters) squared. With the participant in a seated position ≥ 3 blood pressure measurements were taken 5 to 10 minutes apart and the average blood pressure was used in these analyses. Hypertension was defined as blood pressure $>140/90$ mm Hg, self-reported diagnosis of hypertension, or use of antihypertensive medications. Diabetes was diagnosed based on American Diabetes Association criteria or current use of oral hypoglycemic agents or insulin. Stable coronary artery disease was defined as patients with angiographically established coronary disease excluding those with effort angina, unstable angina, and acute myocardial infarction in the previous 3 months. Electrocardiography was performed in all participants to evaluate for changes that would suggest ischemic heart disease. In addition to stable obstructive coronary artery disease, patients from any site with previous angina, chronic stable angina, or previous myocardial in-

Departments of [a]Nephrology and [b]Biochemistry, Muljibhai Patel Urological Hospital, Nadiad, India; [c]University of Arkansas for Medical Sciences, Little Rock, Arkansas; [d]Central Arkansas Veterans Healthcare System, Little Rock, Arkansas; [e]Bhailal Amin General Hospital, Vadodara, India. Manuscript received August 1, 2011; revised manuscript received and accepted September 13, 2011.

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*Corresponding author: Tel: 501-257-5832; fax: 501-257-5827.

E-mail address: shahsudhirv@uams.edu (S.V. Shah).

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Table 1
Clinical characteristics overall and for those in upper one-third and lower two-thirds of serum catalytic iron

		Serum Catalytic Iron		p Value†
	Full Sample (n = 496)	Upper 1/3 (n = 165)*	Lower 2/3 (n = 331)*	
Age (years)	45 ± 12	51 ± 13	43 ± 11	<0.0001
Men	85%	81%	86%	0.16
Systolic blood pressure (mm Hg)	126 ± 14	131 ± 20	124 ± 9	0.0001
Total cholesterol (mg/dl)	197 ± 74	195 ± 80	199 ± 71	0.007
Low-density lipoprotein (mg/dl)	125 ± 63	127 ± 97	124 ± 38	0.003
High-density lipoprotein (mg/dl)	44.1 ± 10.2	40.6 ± 13.7	45.9 ± 7.4	<0.0001
Triglycerides (mg/dl)	138 ± 80	170 ± 81	122 ± 75	<0.0001
High-sensitivity C-reactive protein (mg/L)	1.7 (0.7–4.9)	2.4 (1.0–10.3)	1.4 (0.6–3.4)	<0.0001
Serum creatinine (mg/dl)	0.9 (0.8–1.0)	1.0 (0.8–1.7)	0.9 (0.8–1.0)	<0.0001
Ferritin (ng/ml)	50 (30–92)	61 (38–135)	44 (26–80)	<0.0001
Estimated glomerular filtration rate (ml/min/1.73 m^2)	88.4 ± 31.0	70.6 ± 34.5	97.3 ± 24.8	<0.0001
Diagnosis				
Cardiovascular disease	17%	39%	6.00%	<0.0001
Chronic kidney disease	51%	75%	38%	<0.0001
Diabetes mellitus	17%	32%	9.40%	<0.0001
Hypertension	25%	49%	14%	<0.0001
Obesity (body mass index ≥30 kg/m^2)	7.10%	10%	5.40%	0.05
Uses tobacco	24%	21%	26%	0.21

* Number available except for ferritin, where only 152 and 291 were available for the upper 1/3 versus the lower 2/3, respectively.
† Wilcoxon rank-sum test for continuous variables and chi-square test for categorical variables indicating differences of characteristics across the upper 1/3 versus the lower 2/3 of catalytic iron.

farction were included as subjects with CVD. CKD was defined from National Kidney Foundation KDOQI guidelines (2006) based on estimated glomerular filtration rate (eGFR), proteinuria, and structural damage. For men eGFR was calculated by the Cockcroft-Gault equation: eGFR = (140 – age) × weight (kilograms)/72 × serum creatinine (milligrams per deciliter); for women the value was multiplied by 0.85. Framingham Risk Score was defined based on the National Cholesterol Education Program definition of 10-year CHD risk,[14] where any tobacco use was substituted for smoking.

Participants had blood drawn after fasting for ≥12 hours. Measurements of blood glucose, lipid profiles, serum ferritin, and high-sensitivity C-reactive protein were performed on a fully automated biochemistry analyzer. Serum creatinine was measured by the Jaffe kinetic reaction with alkaline picric acid using a kit prepared by Erba Diagnostics Mannheim (Mumbai, India).

Catalytic iron was measured from serum using the bleomycin-detectable iron assay as described previously.[15,16] Briefly, the assay uses bleomycin, which binds to and degrades DNA in the presence of labile iron, forming a product that reacts with thiobarbituric acid to form a chromogen. To avoid external iron contamination, reactions were carried out in disposable polypropylene tubes and all soluble reagents except bleomycin were treated overnight with Chelex (Bio-Rad Laboratories India Pvt. Ltd., Mumbai, India) (300 mg for a 10-mL solution). Intra-assay coefficients of variation for serum bleomycin-detectable iron (catalytic iron) for low (mean 0.04 μmol/L), medium (mean 0.57 μmol/L), and high (mean 3.15 μmol/L) levels of catalytic iron were 8.1%, 8.8%, and 4.0%, respectively, whereas interassay coefficients of variation were 13.0%, 14.9%, and 10.7%, respectively.

Serum catalytic iron was log-transformed or categorized into thirds using SAS (SAS Institute, Cary, North Carolina) quantiles because of markedly skewed distribution. Subjects in the lowest 2/3 of catalytic iron formed the reference group because of the very low CVD prevalence in the lowest third. Boxplots depicted the distribution of serum catalytic iron on the log scale for the overall sample and for participants with any and those without all the co-morbid conditions in the present study (diabetes mellitus, CKD, CVD, and hypertension). Differences in clinical characteristics for participants in the upper 1/3 versus the lower 2/3 of catalytic iron were tested with chi-square tests (categorical variables) and with Wilcoxon rank-sum tests (continuous variables). Logistic regression models tested associations between serum catalytic iron and CVD. To control for important clinical characteristics, multivariable models were developed using purposeful selection[17] with a statistical significance criterion of a p value ≤0.05 (Wald test for covariates and deviance test for interactions). Covariates were tested for confounding and were retained if they produced an ≥15% change in the odds ratio (OR) for level of catalytic iron. Because of marked correlation with major disease categories and risk factors, center effects could not be tested in logistic models. Possible interactions between serum catalytic iron and major clinical characteristics identified initially by Breslow-Day tests of homogeneity were evaluated by the deviance test in the fully adjusted logistic model. SAS 9.2 was used in all analyses.

Results

This middle-aged population of mostly men had several co-morbid conditions (Table 1). Prevalences of CVD for the highest to lowest thirds of catalytic iron were 39%, 8.6%,

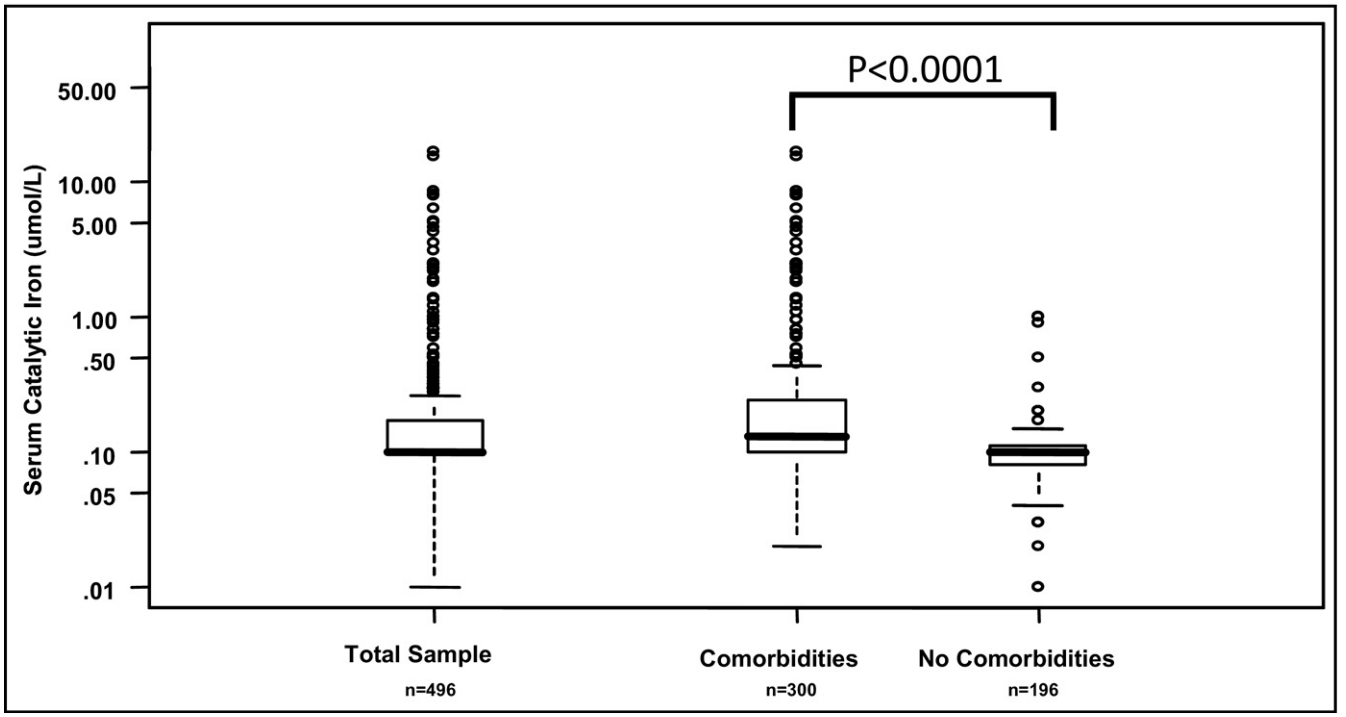

Figure 1. Boxplots for serum catalytic iron overall and by co-morbidity status (presence of diabetes mellitus, chronic kidney disease, cardiovascular disease, or hypertension vs absence of these co-morbidities) using a log scale *(y axis)*. First to third quartiles *(box heights)*, medians *(horizontal lines within boxes)*, quartiles with the smallest *(whiskers above boxes)* and largest *(whiskers below boxes)* values within 1.5 times the interquartile range, and outliers *(circles)* are depicted.

Table 2
Serum catalytic iron medians and interquartile ranges by patient co-morbidities and age

Catalytic Iron (μmol/L)	Age (years)		CVD		CKD		Diabetes Mellitus		Hypertension	
	≤62 (n = 459)	>62 (n = 37)	Yes (n = 85)	No (n = 411)	Yes (n = 251)	No (n = 245)	Yes (n = 83)	No (n = 413)	Yes (n = 126)	No (n = 370)
Median	0.1	0.20*	0.21	0.10*	0.13	0.10*	0.19	0.10*	0.2	0.10*
Interquartile range	0.10–0.15	0.14–0.32	0.14–0.29	0.09–0.13	0.10–0.25	0.08–0.12	0.11–0.40	0.10–0.14	0.11–0.40	0.09–0.13

* All p values are <0.0001 for Wilcoxon rank-sum tests comparing medians for those with and without each characteristic.

and 0.9%. Therefore, to have sufficient events in the reference group, we combined subjects within the middle and lower thirds of catalytic iron. Several significant detrimental associations were found for subjects in the upper third of serum catalytic iron (range 0.14 to 16.7 μmol/L) compared to lower levels (range 0.01 to 0.13 μmol/L): they were older and had higher systolic blood pressure, abnormal lipid levels (lower high-density lipoprotein, higher low-density lipoprotein, and higher triglycerides), higher high-sensitivity C-reactive protein, higher serum creatinine, higher ferritin, lower eGFR, and higher prevalences of co-morbidities (CVD, CKD, diabetes mellitus, hypertension, and obesity; Table 1). No significant differences in gender or prevalence of tobacco use were found for level of catalytic iron. However, the limited numbers of women could have prevented identification of gender differences.

In the overall sample, the median (interquartile range) for serum catalytic iron was 0.1 μmol/L (0.1 to 0.17; Figure 1). Subjects with ≥1 co-morbidity (diabetes, hypertension, CKD, or CVD) had significantly higher catalytic iron (median 0.13 μmol/L, interquartile range 0.1 to 0.24) than subjects with no co-morbidity (median 0.1 μmol/L, interquartile range 0.08 to 0.11, p <0.0001; Figure 1). Median serum catalytic iron was significantly higher for patients >62 years old compared to younger subjects and for subjects with (compared to those without) CVD, CKD, diabetes mellitus, and hypertension (Table 2).

In the final analytic sample, the odds of having existing CVD was 10 times as high for subjects in the upper third of catalytic iron compared to those in the lower 2/3 in unadjusted analyses (Table 3). Adjusting for age decreased the OR by >1/2 (OR 4.9, confidence interval 2.5 to 9.3), but the association remained highly significant (p <0.0001). Additional adjustment for gender had minimal impact on the association (Table 3). Although the Breslow-Day test for homogeneity of effects found potential interactions between levels of catalytic iron and diabetes mellitus, CKD, hypertension, and age category, no significant interaction was found in fully adjusted logistic regression models. In the final model that adjusted for age, Framingham Risk Score, eGFR, hypertension status, high-density lipoprotein cholesterol, and systolic blood pressure, the OR was decreased to

Table 3
Odds ratios and 95% confidence intervals indicating association for upper one-third versus lower two-thirds of serum catalytic iron and existing cardiovascular disease

Model (n = 496)	Level of Catalytic Iron	OR (95% CI)	p Value*
Unadjusted	Upper 1/3	10.1 (5.8–17.5)	<0.0001
	Lower 2/3	1	
Adjusted for age, gender	Upper 1/3	4.9 (2.6–9.5)	<0.0001
	Lower 2/3	1	
Full model†	Upper 1/3	3.8 (1.4–10.1)	<0.0072
	Lower 2/3	1	

* Wald chi-square test for upper 1/3 versus lower 2/3 for serum catalytic iron from logistic regression models.

† Adjusted for age, Framingham 10-year coronary heart disease risk score, estimated glomerular filtration rate, high-density lipoprotein cholesterol, systolic blood pressure, and hypertension.

CI = confidence interval.

3.8, but the association remained significant (Table 3). Testing the log-transformed catalytic iron in the logistic models yielded results that were consistent with those based on categorical catalytic iron; significant associations with CVD were found in analyses mirroring those listed in Table 3. After adjustment for age, Framingham Risk Score, eGFR, hypertension status, high-density lipoprotein cholesterol, and systolic blood pressure, the OR was 1.8 (95% confidence interval 1.4 to 2.2, p <0.0001) for each unit increase. However, the association was nonlinear because the addition of the squared term was significant in analyses. Therefore, we focused on the results based on categorical catalytic iron.

Discussion

In this study we report a direct association between serum catalytic iron and prevalent CVD after controlling for multiple known risk factors for atherosclerosis. Several of these risk factors were associated with serum catalytic iron in bivariate analyses. However, we acknowledge several study limitations. We used a convenience sample including 17% with stable CHD for this cross-sectional exploratory analysis. To understand the direction of the association between catalytic iron and CVD in the general population would require additional prospective studies. Another potential issue for our study and other studies is that a single measurement of catalytic iron is unlikely to reflect the overall long-term exposure to reactive catalytic iron.[13] This may have resulted in the lack of a detrimental association in 1 study of incident CHD.[5] Furthermore, because of the small sample, we were unable to explore completely several potential interactions that suggested that catalytic iron associations might vary depending on the presence or absence of some diseases and possibly by age. Although associations with various types of CVD are of interest, our participants with CVD were almost exclusively patient with stable CHD, preventing evaluation of other categories of vascular disease. In addition, the Framingham 10-year CHD risk score is known to be suboptimal for representing CHD risk in patients with CKD. Nonetheless, one of the striking findings was that the unadjusted odds of having existing CVD for subjects in the upper third of serum catalytic iron was 10 times that for subjects with lower levels. Even after adjustments for important clinical characteristics, the OR remained high and significant at 3.8. This is in keeping with previous studies that demonstrated the presence of iron in human atherosclerotic plaques[18–20] and with the association of catalytic iron with acute coronary syndrome.[16]

A direct association between increased iron stores and atherosclerotic CVD is still controversial.[3] As reviewed, the iron-heart hypothesis was first postulated by Sullivan[21] in the early 1980s. He suggested that the lower incidence of CHD in premenopausal women compared to men of the same age is attributable to lower body iron stores caused by regular blood loss. However, epidemiologic studies assessing serum ferritin or its change as an indicator of iron stores have provided conflicting results, with some studies reporting a positive association with atherosclerotic disease or its progression[11,22–24] and others, including a meta-analysis of incident CHD, reporting no association.[2,25–27] Some inconsistencies may have resulted because the iron in ferritin is bound, and only free or loosely bound iron participates in redox reactions[15] that produce free radicals in biological samples. It is important that total body iron is not consistently related to the level of biologically active iron[12,13] and that many studies evaluating ferritin may not have accounted completely for confounding by inflammation.[22–27]

Demonstration that removal of catalytic iron results in clinical benefit would provide evidence for the role of catalytic iron in CVD. It should be noted that the first randomized multicenter trial (Iron [Fe] and Atherosclerosis Study [FeAST]) reported no significant benefit in all-cause mortality or nonfatal myocardial infarction in patients who underwent a decrease in iron stores by phlebotomy.[28] Sullivan[29] argued that, among other reasons, the FeAST trial may have failed because the study design did not achieve full iron depletion. Nevertheless, in our opinion, the study results were not surprising because iron status does not reflect the iron available to catalyze free-radical reactions as we previously reported in an animal study.[12] An iron-deficient diet resulted in low total body iron but had no effect on catalytic iron in the kidney cortex after ischemia-reperfusion injury. Thus, iron status per se may not dictate susceptibility to injury but rather iron that is catalytically available to participate in free radical reactions. Iron may contribute to atherosclerosis by affecting several biological processes relevant to atherosclerosis[6,20,30] and by impairing vascular function.[30] Therapeutic trials would provide additional evidence for a cause–effect relation between catalytic iron and CVD.

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