Ferritin levels, inflammatory biomarkers, and mortality in peripheral arterial disease: A substudy of the Iron (Fe) and Atherosclerosis Study (FeAST) Trial

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Background: **This study delineated correlations between ferritin, inflammatory biomarkers, and mortality in a cohort of 100 cancer-free patients with peripheral arterial disease (PAD) participating in the Veterans Affairs (VA) Cooperative Study #410, the Iron (Fe) and Atherosclerosis Study (FeAST). FeAST, a prospective, randomized, single-blind clinical trial, tested the hypothesis that reduction of iron stores using phlebotomy would influence clinical outcomes in 1227 PAD patients randomized to iron reduction or control groups. The effects of statin administration were also examined in the Sierra Nevada Health Care (SNHC) cohort by measuring serum ferritin levels at entry and during the 6-year study period. No difference was documented between treatment groups in all-cause mortality and secondary outcomes of death plus nonfatal myocardial infarction and stroke. Iron reduction in the main study caused a significant age-related improvement in cardiovascular disease outcomes, new cancer diagnoses, and cancer-specific death.**

Methods: **Tumor necrosis factor (TNF)-α, TNF-α receptors 1 and 2, interleukin (IL)-2, IL-6, IL-10, and high-sensitivity C reactive protein (hs-CRP) were measured at entry and at 6-month intervals for 6 years. Average levels of ferritin and lipids at entry and at the end of the study were compared. The clinical course and ferritin levels of 23 participants who died during the study were reviewed.**

Results: **At entry, mean age of entry was 67 ± 9 years for the SNHCS cohort, comparable to FeAST and clinical and laboratory parameters were equivalent in substudy participants randomized to iron reduction (n = 51) or control (n = 49). At baseline, 53 participants on statins had slightly lower mean entry-level ferritin values (114.06 ng/mL; 95% confidence interval [CI] 93.43-134.69) vs the 47 off statins (127.62 ng/mL; 95% CI, 103.21-152.02). Longitudinal analysis of follow-up data, after adjusting for the phlebotomy treatment effect, showed that statin use was associated with significantly lower ferritin levels (−29.78 ng/mL; Cohen effect size, −0.47 [$t_{df,\ 134} = 2.33$, $P = .02$]). Mean follow-up average ferritin levels were higher in 23 participants who died (132.5 ng/mL; 95% CI, 79.36-185.66) vs 77 survivors (83.6 ng/mL; 95% CI, 70.34-96.90; Wilcoxon $P = .05$). Mean follow-up IL-6 levels were higher in dead participants (21.68 ng/mL; 95% CI, 13.71-29.66) vs survivors (12.61 ng/mL; 95% CI, 10.72-14.50; Wilcoxon $P = .018$). Ferritin levels correlated (Pearson) with average IL-6 levels ($r = 0.1845$; $P = .002$) and hsCRP levels ($r = .1175$; $P = .04$) during the study.**

Conclusion: **These data demonstrate statistical correlations between levels of ferritin, inflammatory biomarkers, and mortality in this subset of patients with PAD. (J Vasc Surg 2010;51:1498-503.)**

Clinical Relevance: **This research examined relationships between iron storage and inflammatory biomarkers in a cohort of 100 cancer-free PAD patients with peripheral arterial disease from the Veterans Affairs (VA) Sierra Nevada Health Care System (SNHCS) cohort participating in a prospective, randomized, single-blind, clinical trial to test the hypothesis that iron in excess of physiologic requirements promotes atherosclerosis. Calibrated phlebotomy was used to reduce iron storage in risk factor-matched participants. The main study, VA Cooperative Study (CSP) 410, demonstrated clinical benefits in reduction of death from primary and secondary end points for participants aged between 43 and 61 years. This SNHCS substudy demonstrated significant relationships between total body iron stores as determined by ferritin, inflammatory cytokines, and high-sensitivity C-reactive protein (hsCRP). Ferritin levels positively correlated with**

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interleukin-6 levels and hsCRP levels, whereas ferritin and interleukin-6 levels were significantly higher in participants who died vs survivors. Statin administration reduced ferritin levels independently of phlebotomy, indicating an effect of these agents on iron metabolism. Our findings support a biologic rationale for measurement of serial ferritin levels in patients with atherosclerosis. Because iron-induced oxidative stress contributes to inflammatory responses, determination of optimal iron marker levels to be maintained by calibrated phlebotomy is a clinically relevant concept for future outcome studies.

This report describes correlations between ferritin levels, inflammatory biomarkers, and mortality at entry and at 6-month follow-up intervals during the 6-year study duration obtained from 100 consenting Sierra Nevada Health Care (SHNC) participants in the Iron (Fe) and Atherosclerosis Study Study (FeAST), Veterans Affairs (VA) Cooperative Study (CSP) #410. The FeAST study tested the concept that iron-induced oxidative stress might provide inflammatory stimuli in atherosclerosis.[1-11] Relationships between iron status (ferritin levels) and inflammatory markers at entry before randomization and during follow-up were explored in the SNHCS subset participants regardless of randomization status.

The FeAST trial (1999 to 2005)[12] used phlebotomy to test the iron hypothesis. The trial randomized 1277 previously cancer-free participants with advanced but stable peripheral arterial disease (PAD) from 24 VA Medical Centers to graded reduction of iron stores by calibrated phlebotomy vs a nonphlebotomy control group. Details of study design and execution have been reported.[12] Randomization variables at entry included age, ferritin and cholesterol levels, smoking status, and diabetes status. The primary outcome was all-cause mortality and the secondary outcome was death plus nonfatal myocardial infarction and stroke. Data on cardiovascular disease (CVD) outcomes and on the occurrence of new cancer diagnoses and cancer-specific deaths were collected prospectively and analyzed on intent-to-treat basis.

Overall, no significant differences between treatment groups for primary or secondary end points were detected. However, secondary analysis by randomization variables showed that age interacted significantly with treatment. The hazard ratios from the Cox proportional hazard regression model were used to compare primary and secondary end points. Significant improvement with younger age at entry was observed for the secondary end point ($P = .004$ for interaction). Significant improvement in primary ($P = .02$) and secondary ($P < .001$) end points was observed in the youngest age quartile participants (age 43 to 61) randomized to iron reduction compared with control.[12] Iron reduction resulted in a 37% lower incidence of new cancer diagnoses ($P = .025$), reduced risk of new cancer by Kaplan-Meier ($P = .04$) and cumulative incidence analysis ($P = .02$), and reduced cancer-specific mortality ($P = .003$) and all-cause mortality in participants diagnosed with cancer ($P = .009$).[13] Risk reduction began ≤6 months, correlated with compliance, and occurred across the entire age spectrum. Minimal cancer risk was observed, with mean follow-up ferritin levels <57 ng/mL.[13]

A prior SNHCS exploratory study demonstrated elevated levels of inflammatory cytokines in PAD participants compared with controls along with effects of phlebotomy at 6 months on ferritin levels and reduction of inflammatory cytokines in the upper quartile of values.[14] The present substudy tracked relationships between average levels of ferritin, cytokines, and high-sensitive C-reactive protein (hsCRP), and statin use in the SNHCS cohort during the 6 years of the trial.

METHODS

All investigations in this study were performed according to protocols approved by the VA SNHCS Research and Development Committee and the University of Nevada Biomedical Instructional Review Board, including retrospective use of serum samples after death.

From 1999 to 2005, the study monitored 100 participants from the VA SNHCS who were a mean age of 67 years (range, 45-82). Of these, 51 were randomized to phlebotomy and 49 to a control group. Mortality outcomes were recorded according to intent-to-treat, age at death, and cause of death.

Calibrated phlebotomy was performed according to the formula used in the FeAST overall study.[12] (Ferritin ng/mL −25) × 10 = mL blood to be removed. The primary and secondary age-related end points for the SNHCS cohort were calculated in a similar manner to that used in the overall FeAST study using Kaplan-Meier comparisons. The Cox proportional hazard regression model was used to compute hazard ratios (HR) and 95% confidence intervals (CI), with adjustment to covariates for analyses of primary and secondary outcomes. No significant differences were detected in the SNHCS cohort.

The proportion of participants receiving statins was tracked throughout the study. The effect of statins on ferritin levels was assessed using a longitudinal multivariate model SAS PROC MIXED[15] for the SNHCS cohort and for all the FeAST main study participants. The model tested the main effects using follow-up ferritin as the dependent variable. Independent variables were phlebotomy (iron reduction vs control), statin use at each follow-up, and other baseline covariates. Biomarkers measurements, including ferritin, cytokines, and hsCRP, were analyzed by methods previously described.[14,16] Inflammatory cytokines tumor necrosis factor (TNF)α, TNFα-R1 and R2, interleukin (IL)-6, and anti-inflammatory cytokines interleukin (IL)-2 and IL-10 were analyzed by enzyme-linked immunosorbent assay at baseline and every 6 months and correlated with ferritin levels in all participants, as described in prior reports.[14,16] Because cytokine values are not normally dis-

tributed, the Wilcoxon rank-sum test was used for these statistical analyses. Baseline average levels of ferritin, cholesterol, low-density lipoprotein, high-density lipoprotein, and triglycerides in control and intervention groups were compared at entry and at 6-month intervals to the end of the trial using the Wilcoxon rank-sum method. The entry and final values were tabulated.

RESULTS

Twenty-three participants died during the study: 12 among the 51 assigned to the iron-reduction group and 11 among the 49 in the control group. The causes of death in the iron-reduction group were cancer in 3, CVD in 6, and other (eg, trauma) in 3; and in the control group were cancer in 2, CVD in 5, and other causes in 4. The primary and secondary end points were similar to those in the main study using identical analyses. Three of the 51 participants randomized to phlebotomy subsequently declined this intervention, resulting in a 94% compliance rate.

The maximum ferritin reduction occurred between baseline levels and the first 6-month follow-up value, after which ferritin levels tended to plateau. By intent-to-treat analysis, the time to death was 21 to 65 months in the 12 patients in the iron-reduction group and 5 to 49 months in the control group. The Cox proportional hazards model was used to analyze survival times. The HR were 1.005 (95% CI, 0.443-2.280; $P = .9896$) for deaths and 1.205 (95% CI, 0.598-2.425; $P = .602$) for secondary events in the SNHS cohort. The difference between the control and phlebotomy groups was not significant.

The association between death and elevated ferritin levels and inflammatory cytokines was related to the average of values during the entire 6-year study. The time between the last ferritin levels obtained in stable SNHS patients, according to the FeAST protocol was an average of 245 days (median, 124; range, 3-1583 days). An 81-year-old patient, who was a noncompliant participant in the intervention group, died after bronchoscopy 3 days after a ferritin determination of 300 ng/mL. After review of all entry and terminal ferritin levels among the 23 deaths, only this single instance would represent an acute-phase reaction involving an elevated ferritin level.

FeAST protocols required exclusion of participants with a history of malignancy, disturbance in iron metabolism, hepatic or renal abnormalities, recent infection, antibiotic administration, or bleeding event because such overt conditions are capable of affecting ferritin levels. If ferritin levels were elevated in a participant who had an infection or inflammatory process, a ferritin level was repeated after the infection or inflammatory process subsided and only then was phlebotomy performed. Phlebotomy was withheld for those who refused and for those in unstable conditions.

To examine the possibility that ferritin levels and biomarkers were possibly related to underlying disease states, Pearson correlation coefficients were used to examine relationships between serum measures and body mass index (BMI), diabetes, and smoking. A direct relationship between ferritin and IL-6 levels was observed that was maintained after correction for BMI, diabetes, and smoking status in all participants and survivors. The observed relationship between ferritin and CRP levels was maintained after corrections for BMI, diabetes, and smoking status only in survivors.

Table I. Mean follow-up ferritin levels and intervention during study period in survivors and nonsurvivors in the Sierra Nevada Health Care cohort of the Veterans Affairs Cooperative Study #410

Treatment group	*Participants*	*Ferritin level*	P[a]
	No.	Mean ± SD, ng/mL	
Control			.60
Survivors	38	111.1 ± 65	
Nonsurvivors	11	159.5 ± 158.3	
Iron reduction			.05
Survivors	39	56.2 ± 29.9	
Nonsurvivors	12	112.3 ± 74.2	
Total			.05
Survivors	77	83.6 ± 57.3	
Nonsurvivors	23	132.5 ± 116.8	

SD, Standard deviation.
[a]Analyzed by the Wilcoxon test.

Table I reports the relationship between the mean ferritin level during the study period and mortality for each intervention group and for patients combined; the data were analyzed using the Wilcoxon test. The mean ferritin levels during the entire follow-up period in the 23 nonsurviors was 132.5 ± 116.8 ng/mL, which was significantly higher than the level of 83.6 ± 57.3 ng/mL in the survivors (Wilcoxon, $P = .05$). Thus, the average ferritin level appeared to be directly related both to mortality and levels of inflammatory biomarkers.

An important confounder addressed in the current substudy related to statin use, which was not a criterion for randomization into FeAST. At baseline, 47 participants not receiving statins exhibited a trend toward higher mean ferritin levels of 127.6 ng/mL (95% CI, 103.21-152.02) compared to 114.06 ng/mL (95% CI 93.42-134.69) in the 53 who were receiving statins. This difference was not statistically significant. During the study, however, 31 more participants began taking statins, and these agents were discontinued in 18 participants at different times. Longitudinal analysis (SAS PROC MIXED)[15] of all follow-up data showed, after adjusting for the phlebotomy treatment effect, that statin administration had a significant effect on the reduction of ferritin levels (−29.78 ng/mL) with a Cohen effect size of −0.47 ($t_{df = 134} = -2.33$; $P = .02$).

Table II reports the Pearson correlation coefficients between levels of ferritin, cytokines, and hsCRP in the 100 participants, including the 77 survivors and 23 nonsurvivors. Significant relationships were found between levels of ferritin and IL-6 ($r = 0.1845$, $P = .002$) and hsCRP ($r = 0.12$, $P = .04$). Ferritin levels correlated with IL-6 levels in surviving participants ($r = 0.21$, $P = .002$), but IL-6 and ferritin were significantly higher in participants who died than in survivors. Mean follow-up

Table II. Pearson correlation coefficients (*r*) between ferritin levels, cytokines, and biomarkers during the study period in Veterans Affairs Sierra Nevada Health Care cohort of Cooperative Study #410

Group	*IL6*	*CRP*	*TNFa*	*TNFa R1*	*TNFa R2*	*IL-2*	*IL-10*
All participants (N = 100)							
Ferritin							
r	0.18	0.12	−0.04	−0.02	−0.04	0.05	0.04
P	.002	.04	.48	.76	.50	.46	.48
Survivors (n = 77)							
Ferritin							
r	0.21	0.16	−0.02	−0.04	0.01	0.02	−0.20
P	.002	.01	.78	.54	.85	.16	.77
Nonsurvivors (n = 23)							
Ferritin							
r	0.10	−0.05	−0.15	−0.006	−.24	−0.07	0.09
P	.47	.68	.22	.96	.05	.06	.48

CRP, C-reactive protein; *IL*, interleukin; *TNF-α*, tumor necrosis factor α.

Table III. Correlations during the study period between C-reactive protein and tumor necrosis factor-α (TNF-α) receptors in Veterans Affairs Sierra Nevada Health Care cohort of Cooperative Study #410

		TFN-α R1		*TNF-α R2*	
Group	*No.*	r	P	r	P
All participants	100	0.17	.003	0.38	<.0001
Survivors	77	0.20	.003	0.41	<.0001
Nonsurvivors	23	0.10	.45	0.19	.15

IL-6 levels were 21.68 ng/mL (95% CI 13.71-29.66) in the 23 participants who died compared with 12.61 ng/mL in the 77 survivors (95% CI 10.72-14.50; $P = .02$). No correlations were found among ferritin levels, TNF-α, IL- 2, and IL-10.

Ferritin levels correlated (Pearson) with hsCRP levels ($r = 0.12$, $P = .04$) for all participants and for survivors ($r = 0.16$, $P = .01$). CRP is of interest because this is the commonly accepted clinical biomarker for gauging an inflammatory response. Table III reports CRP level correlations with TNFα-R1 ($r = 0.17$, $P = .003$) and R2 ($r = 0.386$, $P \leq .0001$) for all participants and for survivors for R1 ($r = 0.20$, $P = .002$) and R2 ($r = 0.41$, $P \leq .0001$). No correlation between CRP and ferritin levels was detected in the 23 participants who died.

Table IV summarizes Wilcoxon rank-sum testing for the average baseline ferritin levels before intervention, which were comparable at 116 ± 73.1 ng/mL in the control group and 124 ± 85 ng/mL in the iron-reduction group ($P = .99$). Ferritin levels at the point of last follow-up and lipid values are included to compare differences. At the last follow-up, ferritin levels in controls averaged 120.5 ± 90.6 ng/mL and 69.9 ± 50.3 ng/mL in the iron-reduction group ($P = .0003$). No significant differences occurred between entry values and last follow-up values for cholesterol, high-density and low-density lipoprotein, and triglycerides.

Table IV. Initial average ferritin and lipid levels at baseline and at last follow-up in the Veterans Affairs Sierra Nevada Health Care cohort of Cooperative Study #410

Variable	*Control*	*Iron reduction*	P[a]
Ferritin, ng/mL			
Baseline	116.4 ± 73.1	124.2 ± 84.5	
Follow-up	120.5 ± 90.6	69.9 ± 50.3	
Difference	5.6 ± 66.9	−53.3 ± 88.2	.0001
Cholesterol, mg/dL			
Baseline	187.4 ± 37.6	197.1 ± 39.7	
Follow-up	184.2 ± 30.2	185.1 ± 29.5	
Difference	−3.2 ± 32.7	−11.7 ± 34.1	.63
HDL, mg/dL			
Baseline	42.6 ± 17.6	45.8 ± 14.5	
Follow-up	40.3 ± 16.9	45.3 ± 15.7	
Difference	−2.4 ± 7.9	−0.7 ± 9.5	.54
LDL, mg/dL			
Baseline	118.6 ± 33.6	125 ± 33.5	
Follow-up	115.5 ± 24.9	113.4 ± 27.3	
Difference	−2.3 ± 27	−9.7 ± 28.4	.46
Triglycerides, mg/dL			
Baseline	175.7 ± 134.5	171.6 ± 86.2	
Follow-up	175.3 ± 94.7	165.1 ± 105.8	
Difference	2.5 ± 92.5	−7.8 ± 81.6	.12

LDL, Low-density lipoprotein; *HDL*, high-density lipoprotein.
[a]Analyzed by the Wilcoxon test.

DISCUSSION

This study demonstrated an association between levels of ferritin, inflammatory cytokines, and hsCRP in a subset of 100 individuals with PAD during a clinical trial of iron reduction. Overall age at entry and mortality in the SNHCS cohort during the trial paralleled the overall results of the main FeAST trial of 1277 participants.[12] Levels varied widely in both the iron-reduction and control groups; the intervention group overall showed an average terminal reduction of ferritin levels to about 70 mL. However, statistical correlations between ferritin, inflammatory biomarkers, and death occurred during the course of the trial irrespective of intervention assignments. These data derive

from a subset of participants of a randomized trial of phlebotomy intended to deliberately lower ferritin levels, which as in the main trial, did not affect overall mortality. However, the data in the present study highlight compliance issues related to achieved ferritin levels, related inflammatory cytokine levels, and mortality that occurred regardless of allocation to phlebotomy or control groups.

These correlations appear to provide biologic support to the previously reported secondary analyses of the main FeAST trial during which secondary data analyses demonstrated participants aged 43 to 61 years received major benefits.[12] The predictive value of IL-6 for death in the present study was a notable finding. This inflammatory cytokine appeared as a highly specific marker for death. CRP alone did not predict death in the SNHCS cohort or differentiate between participants who were or were not receiving statins. In addition, hsCRP levels did not differ between survivors and nonsurvivors, participants randomized to control or intervention groups, or between diabetic and nondiabetic participants. These discordances may relate to the effect of small sample size, and further studies are needed. Our prior report[14] in 2003 demonstrated a strong relationship between advanced PAD and elevated hsCRP and inflammatory cytokine levels compared with healthy controls studied at the beginning of the FeAST trial.

Relationships of statin administration to biomarkers at the time of entrance into FeAST before randomization, based on physician preference, were described in a prior report.[16] At that time, 53% of participants entering the study were receiving statins. A significant proportion of these were diabetic patients who also exhibited significantly elevated IL-6 levels that were apparently not reduced by statin use. With subsequent randomization, the direct relationship between ferritin and IL-6 levels was not affected by diabetes or BMI in all participants or in survivors. Evolving prescribing standards during the period of the trial (1999 to 2005) recommended statin use for all patients with PAD. In accordance with practice recommendations for PAD, statin use prescriptions commenced in 31 SNHCS participants, and 18 participants discontinued statins for unspecified reasons. After adjusting for phlebotomy effect, statin use remained associated with significantly lower ferritin levels.

Statin use was not taken into account during randomization for the CSP #410 FeAST trial, and the evolving practice of prescribing statins for PAD during the study interval was likely a confounding factor. The effect of statins in association with reduced ferritin levels is a provocative finding suggesting a possible mechanism of action of these agents on iron metabolism. The mechanism of statin effect on biomarkers and its pleiotropic anti-inflammatory actions requires further study taking into account duration of treatment, dosage, and type of agent.

During the study period, ferritin levels in the phlebotomy group fell from an average of 124.2 to 69.9 ng/mL, measured at 6 months after the preceding phlebotomy. Future trials might aim for sustained lower ferritin levels, such as 25 to 40 ng/mL, throughout the study interval. Such levels might be obtained with ideal compliance with phlebotomy. The present substudy, which showed unequivocally higher ferritin levels in participants who died than in survivors, irrespective of assignment to control or phlebotomy strategy, supports this view.

Interrelationships between iron storage, ferritin levels, and biomarkers are multiple, complex, and interactive. These require further delineation using prospective, randomized studies sufficiently powered to demonstrate specific subgroup correlations, biomarker profiles, and outcome effects. Selection of younger participants, along with a more robust continuum of reduced ferritin levels into prospective trials, appears desirable. The finding of statistically significant correlations between ferritin and high levels of inflammatory cytokines IL6 and TNF-α R1 and R2 suggests biologic relationships between increased ferritin levels and the inflammatory biomarker responses associated with atherosclerosis and its complications. Ferritin in stable-state patients is considered to be the preferred marker of clinical outcomes that are presumably caused by iron-related stress.[17]

Although causality cannot be inferred from the present study on purely statistical grounds, a recent comprehensive review[18] suggests that poorly liganded iron species lead to the catalytic production of highly damaging pro-oxidant radicals. These are thought to be a major cause of chronic inflammation contributing to vascular and other progressive inflammatory diseases.

More prospective studies are required before iron-reduction strategies for atherosclerosis can become mainstream recommendations. However, the present exploratory study supports a rationale for serial measurements of ferritin levels, not only for assessing the role of iron storage in atherosclerosis but also as a means of screening for iron storage diseases, for example hemochromatosis.

Although the United Blood Service makes no claim for the health benefits of blood donation by healthy individuals, a number of positive reports describe improved health in regular blood donors,[19] who may donate between four to six times yearly with hematocrit in excess of 38%. Some disagree with these observations.[20] The benefits of iron reduction by phlebotomy might prove most favorable for women who do not menstruate and for younger men as well as for other accepted indications to lower iron stores.[19] Additional measures to reduce excess iron stores include avoidance of iron-containing supplements and eating less red meat. Iron supplementation of flour has now been abandoned in Scandinavia.[21] Chelators such as deferasirox[22-23] and other agents are available to reduce iron stores. Vascular specialists might consider measurement of ferritin levels along with assessing levels of lipids and hsCRP. Sequential measurements of IL-6, CRP, and additional inflammatory biomarkers may prove helpful for prognosis and for analyzing biologic events and relating these eventual outcomes.

CONCLUSIONS

Positive statistical correlations between ferritin levels, inflammatory cytokines, CRP, and mortality suggest that iron-induced oxidative stress may relate to inflammatory responses in PAD. The biomarker correlations described in this study provide conceptual support for further trials that investigate reduction of mortality using robust iron reduction strategies.

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AUTHOR CONTRIBUTIONS

Conception and design: RD, VH, PM
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Critical revision of the article: RD, LZ, VH, BC, GS, PM
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