Author's response to reviews

Title: The association of biomarkers of iron status with peripheral arterial disease in US adults

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Author's response to reviews: see over
Dear Editor,

Thank you for your comments and the reviewers’ comments and for the opportunity to submit a revised version of the above mentioned manuscript. We have incorporated the editor’s and reviewers’ comments, which have substantially improved the paper. Please see below for a detailed description of the changes introduced in the manuscript in response to reviewer’s comments. We look forward to the final decision on this manuscript.

Please do not hesitate to contact me if you have additional questions related to this manuscript.

Best regards,

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Response to Reviewer 1:

1. It would be of important to see the results of the analysis before the extensive exclusions were made as well as after the exclusions. Over one-half of the original sample was excluded for various reasons.

Most of the exclusions (44%) were due to exclusion criteria including premenopausal women (due to very few events), anemia (affects iron biomarkers), iron supplements (affects iron biomarkers), likely hemochromatosis (affects iron biomarkers), and having an ABI $\geq 1.3$ (a sign of severe arterial stiffness). Additionally, 16% were excluded due to missing data. Below is a table of the differences between study participants and individuals excluded due to missing data.

<table>
<thead>
<tr>
<th></th>
<th>Men</th>
<th>Women</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Excluded</td>
<td>Included</td>
</tr>
<tr>
<td>Age, y</td>
<td>57 (0.8)</td>
<td>54 (0.3)*</td>
</tr>
<tr>
<td>Non-Hispanic white, %</td>
<td>72 (2.9)</td>
<td>78 (1.8)*</td>
</tr>
<tr>
<td>Non-Hispanic black, %</td>
<td>13 (1.7)</td>
<td>9 (0.9)*</td>
</tr>
<tr>
<td>Mexican-American, %</td>
<td>6 (0.8)</td>
<td>5 (0.8)</td>
</tr>
<tr>
<td>BMI, kg/m$^2$</td>
<td>29 (0.5)</td>
<td>28 (0.2)*</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>208 (2.5)</td>
<td>211 (2.3)</td>
</tr>
<tr>
<td>Diabetes mellitus, %</td>
<td>12 (2.1)</td>
<td>8 (0.6)*</td>
</tr>
</tbody>
</table>

In the manuscript, we have added the following sentence in the Methods section on page 5, paragraph 1, sentence 3:

“When participants excluded because of missing data were compared to those included in the study, excluded participants were more likely to be older, non-Hispanic black, and to have a higher body mass index, lower cholesterol levels, and a higher prevalence of diabetes.”

2. The hemoglobin exclusions may have permitted anemic men but not women to remain in the analyzed sample and should be reconsidered. The lower limit of normal of hemoglobin in Caucasian women is usually 12 g/dL, the level used in the study. The lower limit of normal in Caucasian men is usually 13.5 - 14 g/dL in men whereas the level used in the study was 13. Using different cutoffs for African Americans is appropriate, as the authors have done.

We have altered our definition of anemia to: non-Hispanic black men with hemoglobin $<12.5$ g/dL; other men with hemoglobin $<13.5$ g/dL; non-Hispanic black women with hemoglobin $<11$ g/dL; other women with hemoglobin $<12$ g/dL. As a result, an addition 53 men were excluded. However the results are remarkably similar using this definition of anemia.
We have added the following sentence in the Methods section on page 5, paragraph 1, sentence 2:

“Using alternative cutoff points for excluding participants with anemia did not materially alter the results of the study”.

3. Exclusions or adjustments should include measures of hepatocellular damage. Such damage, which is reflected in elevations in AST and ALT, can profoundly affect serum ferritin concentration and transferrin saturation and may be very common in the population related to such factors as hepatitis C seropositivity (which is often undiagnosed) and heavy alcohol consumption.

We agree that it is important to consider liver damage and have additionally adjusted for ALT (log-transformed, continuous) in multivariable adjusted models. As seen in the tables, adjustment for ALT did not substantially alter the results.

4. Inflammation probably does not fit neatly into the categories of CRP $< 1.0$ mg/dL, and revised approaches to accounting for inflammation should be considered. Part of the solution may be to use CRP as a continuous rather than a dichotomous variable. However, the authors should also consider that hepatitis C, heavy alcohol intake and hepatocellular damage in general all lead to a paradoxical decrease rather than an increase in the CRP.

We changed the way we model CRP in multivariable adjusted models. It is now included as a log-transformed, continuous variable. Also, in response to the previous comment, ALT was added to multivariable adjusted models. The results were markedly similar after these changes.
Response to Reviewer 2:

1) The clinical implication of measuring ferritin/ transferrin saturation in peripheral arterial disease is required in the background.

We added additional context information in the background section of the Abstract. This section of the abstract now reads:

“Several studies have examined the association of biomarkers of iron metabolism with measures of carotid artery atherosclerosis, with inconsistent results. Few studies, however, have evaluated the association between biomarkers of iron metabolism and peripheral arterial disease (PAD). The purpose of this study is to examine the association of ferritin and transferrin saturation with PAD.”

2) In the results, the second sentence is too long; it may be easier for a reader to understand if the sentences are shorter.

We split this sentence into two sentences to make it clearer. It now reads:

“After stratifying by cholesterol levels, the multivariable adjusted odds ratios (95% confidence intervals) for PAD associated with a two-fold increase in ferritin and transferrin saturation was 1.04 (0.78-1.39) and 0.73 (0.35-1.50), respectively, for men with total cholesterol <200 mg/dL and 1.30 (0.99-1.72) and 2.59 (0.99-6.78), respectively, for men with total cholesterol ≥200 mg/dL (p-value for interaction was 0.58 for ferritin and 0.08 for transferrin saturation). After stratifying by cholesterol levels, the multivariable adjusted odds ratios (95% confidence intervals) for PAD associated with a two-fold increase in ferritin and transferrin saturation was 0.66 (0.41-1.05) and 0.75 (0.44-1.28), respectively, for women with total cholesterol <200 mg/dL, and 1.20 (0.95-1.51) and 2.07 (1.01-4.22), respectively, for women with total cholesterol ≥200 mg/dL (p-value for interaction was 0.05 for ferritin and 0.02 for transferrin saturation).”

3) Page 6 Methods: peripheral arterial disease- why was an ABI <0.9 used to define peripheral artery disease? Is this according to the current guidelines?

The use of ABI <0.9 as a cutpoint to define PAD is a generally accepted cutpoint. It is based on a high sensitivity and specificity for detecting angiogram positive disease. We added the following text stating this on page 7, paragraph 1, sentence 7:

“The 0.9 cutoff value for ABI has 95% sensitivity for detecting angiogram positive disease and almost 100% specificity in excluding healthy individuals.”