Author's response to reviews

Title: Inverse correlation between serum interleukin-6 and iron levels among Japanese adults: a cross-sectional study

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Author's response to reviews: see over
Dear Dr O'Donovan,

Please find attached a revised version of our manuscript “Inverse correlation between serum interleukin-6 and iron levels among Japanese adults”, which we would like to resubmit for publication as a Research Article in *BMC Hematology*.

The comments from the reviewers were highly insightful and enabled us to greatly improve the quality of our manuscript. In the following pages, we have summarized point-by-point responses to each of the comments from the reviewers.

Revisions in the text are shown using yellow highlight for additions, and strikethrough (example) for deletions. We hope that the revisions in the manuscript will be sufficient to make our manuscript suitable for publication in *BMC Hematology*.

We look forward to hearing from you at your earliest convenience.

Yours sincerely,

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Responses to the comments of reviewer Kishor B Raja

Comments to the author:

MAJOR;

1. There was no indication of the bleeding procedure i.e. time of draw or if the blood samples were fasting samples? As the authors are no doubt aware, dietary intake as well as timing (diurnal variation) will affect serum iron concentrations. Furthermore, hepcidin concentrations have been reported to exhibit diurnal variation too. The overall process thus needs standardisation (MAJOR REVISION)

Response: We thank the reviewer for pointing out this issue. The subjects in our study included those who visited an urban clinic in Nagoya, Japan to be tested for Helicobacter pylori (H. pylori) infection and subsequent eradication, and they were not requested to fast. Therefore, blood samples were not from fasted subjects. As mentioned by the reviewer, dietary intake could affect serum iron levels. Although some papers have reported that diet has little effect on iron levels [30, 31], we have added this issue as a limitation to the discussion.

As mentioned by the reviewer, hepcidin concentrations show diurnal variation. However, one study showed that in healthy individuals, hepcidin-25 concentrations displayed a diurnal variation, and this pattern was not influenced by food intake [27]. Additionally, in this diurnal variation, hepcidin-25 concentrations were lowest in the early morning and steadily increased throughout the day, before declining during the evening hours. Moreover, some studies have reported that serum iron levels appear to be the highest in the morning, with levels decreasing during the day, and then increasing during the late evening hours [30,31]. Because we collected blood samples at the same time (in the morning) in this study, we believe that an effect of timing of drawing blood did not affect the association between serum iron and IL-6 levels.

We have added a sentence to the Clinical tests section (page 6, line 14):

“All participants provided blood samples in the morning. No fasting was required.”

We have added the following sentences as a limitation to the Discussion (page 15, lines 1-12):

“A limitation of our study is that there was no information of dietary intake that might have affected serum iron levels. Despite fluctuations in dietary iron intake and intermittent losses
through bleeding, plasma iron levels in humans remain stable [26]. While most of the iron entering blood plasma comes from recycling, an appropriate amount of iron is absorbed from the diet to compensate for losses and to maintain nontoxic amounts in stores [26]. However, some studies have reported that diet has little effect on iron levels [30, 31]. In addition, although hepcidin levels show diurnal variation, one study showed that the diurnal variation pattern was not affected by food intake [27]. Therefore, we believe that the lack of information of dietary intake did not considerably affect our findings of a correlation between serum iron and IL-6 levels. Because blood was drawn in the morning in all of the participants, the effect of diurnal variation in serum iron and hepcidin would be small.”

2. Measurement of TIBC and calculation of % transferrin saturation would have been useful in assessing if the changes in serum iron were really significant

Response: We appreciate the reviewer’s comment. According to the reviewer’s comment, we evaluated serum TIBC levels and transferrin saturation (TSAT) (%). Regression analysis adjusted for sex and age showed that lower TSAT (%) was significantly associated with higher log serum IL-6 levels in all subjects ($\beta=-3.31$, $p=0.040$) and in men ($\beta=-5.45$, $p=0.028$), but not in women ($\beta=-1.93$, $p=0.36$). However, in regression analysis adjusted for sex and age, serum TIBC levels were not significantly associated with log$_{10}$ serum IL-6 levels in all subjects ($\beta=3.53$, $p=0.58$).

We have revised the following part in the Methods section (page 6, lines 15–17):

Before revision: Serum iron and IL-6 levels were measured using the Nitroso-PSAP [2-Nitroso-5-(N-propyl-3-sulfopropylamino) phenol] method and CLEIA (chemiluminescence enzyme immunoassay), respectively.

After revision: “Serum iron levels and total iron binding capacity (TIBC) were measured using the 2-nitroso-5-(N-propyl-3-sulfopropylamino) phenol method, and IL-6 levels were determined by chemiluminescence enzyme immunoassay.”

We have added two sentences to the Clinical tests section:

“Transferrin saturation (TSAT) was calculated as serum iron ÷ TIBC ×100.” (page 6, line 18 to page 7 line 1)
“The reference range for serum TIBC was 253–364 µg/dL in men and 246–410 µg/dl in women.” (page 7, lines 8–9)

We have changed several sentences in the Statistical analysis section (page 7, line 15 to page 8, line 2):

**Before revision:**
The strength of association between the logarithm of serum iron levels and the logarithm of serum IL-6 levels was examined using the Pearson correlation coefficient. Multivariate regression analysis was performed to assess the influence of \( \log_{10} \) of IL-6 on the \( \log_{10} \) of serum iron with an adjustment for sex and age (continuous variables).

**After revision:**
“The strength of the associations between the logarithm of serum iron levels, and the logarithm of serum IL-6 levels, serum TIBC levels, and TSAT were examined using the Pearson correlation coefficient. Multivariate regression analysis was performed to assess the effect of \( \log_{10} \) of IL-6 on \( \log_{10} \) of serum iron, TIBC, and TSAT, with adjustment for sex and age (continuous variables).”

We have added the following sentences to the Results section (page 9, line 18 to page 10, line 11):

“Mean serum TIBC levels were 305.7 µg/dL for men (range, 224–431 µg/dL) and 329.9 µg/dL for women (range, 209–501 µg/dL). A low TIBC level was found in 8.6% (n=9) of men and in 1.14% (n=2) of women. A high TIBC level was found in 12.4% (n=14) of men and in 8% (n=15) of women. Serum TIBC levels were not correlated with the logarithm of IL-6 levels overall (r=-0.106, p=0.16). In multiple linear regression analysis adjusted for sex and age, serum TIBC levels were not significantly associated with \( \log_{10} \) serum IL-6 levels in all subjects (\( \beta=3.53 \), p=0.58). Mean TSAT (%) was 39.4 for men (range, 3.0–71.1) and 30.5 for women (range, 3.7–77.6). TSAT (%) was not correlated with the logarithm of IL-6 levels overall (r=-0.036, p=0.55). However, regression analysis adjusted for sex and age showed that a lower TSAT (%) was significantly associated with a higher logarithm of serum IL-6 levels in all subjects (\( \beta=-3.31 \), p=0.040) and in men (\( \beta=-5.45 \), p=0.028), but not in women (\( \beta=-1.93 \), p=0.36).”

We have added the following sentences to the Discussion section (page 13, lines 10–16):

“We found an inverse association between TSAT (%) and the logarithm of serum IL-6 levels in all subjects. However, this association varies between studies, and therefore, further investigations are required on this association. In 34 hemodialysis patients, serum
IL-6 levels were not correlated with TSAT (r=−0.250, p=0.154, n=34) [28]. There was a marginally significant correlation between serum IL-6 levels and TSAT in 34 peritoneal dialysis patients (r=0.067, p=0.078) [29]."

3. The measurement of a positive inflammatory marker (e.g. CRP) in the samples would have been beneficial- to get an indication of the severity of infection? (MAJOR)

Response: We thank the reviewer for the comment. As mentioned by the reviewer, the measurement of inflammatory makers would be useful to determine the severity of infection. Unfortunately, we did not measure any other inflammatory markers (e.g., CRP), except for IL-6 in our study.

We have described this issue as a limitation in the Discussion section (page 15, lines 16 to page 16, line 2).

4. Only 4.3% of the studied group had an IL-6 concentration > 4ng/L. Is there thus too much emphasis on the OR value (7.88) based on such a small patient sample number? (MAJOR)

Response: As pointed out by the reviewer, we only had a small sample with IL-6 levels > 4 pg/mL. Because an IL-6 level > 4 pg/mL is outside of the reference level, we used the cutoff point in our analysis. However, we reanalyzed the data with IL-6 levels ≥3 pg/mL (9.7%, n=27), and the significant association was not altered.

We have added the results of IL-6 levels ≥3 pg/mL to the Results section (page 9, lines 15–17):

“In subjects with an IL-6 level ≥ 3 pg/mL (versus < 3 pg/mL), the OR for low serum iron levels, adjusted for sex, age, and H. pylori infection status, was 8.21 (95% CI 2.0–34.3).”
MINOR:

1. The cut-off levels for IL-6 and iron: were they ascertained in a similar population (in terms of geographical location, dietary intake etc.) to the one being studied? (MINOR)

Response: Both of the cutoff levels were ascertained in the Japanese population.

We have revised part of the Methods section by adding this information (page 7, lines 4 and 7):

Response: We appreciate the reviewer for pointing out this issue.

We have revised part of the Results section (page 9, lines 5-10):

Before revision: However, in the multiple linear regression analysis on log_{10} (serum iron) adjusting for sex and age, lower log_{10} (serum iron) levels were significantly associated with higher log_{10} (serum IL-6) levels in all subjects (β= -0.050, p=0.040), but did not reach statistical significance in males; marginally significant in males (β= -0.059, p=0.073), and not significant in females (β= -0.048, p=0.17).

After revision: “However, in multiple linear regression analysis on log_{10} serum iron adjusted for sex and age, lower log_{10} serum iron levels were significantly associated with higher log_{10} serum IL-6 levels in all subjects (β= -0.050, p=0.040). However, this did not reach statistical significance in males each sex; it was marginally significant in men (β= -0.059, p=0.073) and not significant in women (β= -0.048, p=0.17).”

3. Could the authors comment on whether the fall in serum iron seen in their population could be attributable to (i) iron utilisation by H pylori, (ii) reduction in intestinal iron absorption due to atrophy associated gastric hypochlorhydia- following the infection (iii) heritable differences in hepcidin expression (MINOR)

Response: We thank the reviewer for these suggestions. In the same study subjects as those in the current study, our previous study showed that serum ferritin levels were significantly
lower in H. pylori infected subjects than in uninfected subjects, but serum iron levels were unchanged. Moreover, atrophic gastritis by H. pylori infection was associated with a decrease in ferritin levels, but not with serum iron levels. While ferritin levels represent the body’s iron store, iron levels remain stable because of homeostasis, as so to most of the iron entering blood plasma comes from recycling. Therefore, we speculate that this decrease in serum ferritin levels is attributable to some extent to iron use by H. pylori or a reduction in intestinal iron absorption due to atrophic gastritis following infection. However a decrease in serum iron has not been observed. Although heritable differences in hepcidin expression have been reported, no studies have determined a role of these differences in variation of blood IL-6 levels. Therefore, we cannot comment on such a mechanism of a decrease in iron levels.

This information has been added to the Discussion section (page 14, lines 9–18).

4. There was no indication of assay variability and phenotypic variation in iron metabolism in individuals (MINOR)

**Response:** The coefficient of variability of serum iron was 1.57%. We have added this information to the Methods, and have discussed phenotype variation in iron metabolism in the Discussion section as a limitation.

We have added the following sentence to the Methods section (page 6, lines 17–18).

“The coefficient of variability (CV) of serum iron was 1.57%.”

We have added the following sentences to the Discussion section (page 15, lines 12–16).

“There are heritable differences in hepcidin expression that may determine phenotypic variation in iron metabolism between individuals. Therefore, consideration of phenotypic variation in iron metabolism in individuals is important. Another limitation of our study is that there were no data of phenotypic variation of hepcidin.”
DISCRETIONARY:

i) The significant inverse correlation between the 2 parameters in males (log plots) is, as highlighted by the authors, likely to be due to the 3-4 high IL6 values (DISCRETIONARY)

Response: We thank the reviewer for pointing this out. Although there were several high IL-6 values, the odds ratio of low iron levels for IL-6 levels ≥3 pg/mL (versus <3 pg/mL) also had a significant association for IL-6 levels > 4 pg/mL (versus ≤4 pg/mL). Therefore, we believe that the significant inverse correlation was not likely to be due to the 3–4 high IL-6 values.

Responses to the comments of reviewer Yoko Shibata
Comments to the author:

1. This manuscript demonstrated that inverse correlation between serum interleukin-6 and iron levels among 280 healthy Japanese subjects aged 20-78. However, the authors have not demonstrated blood hemoglobin levels that should be associated with the level of serum Fe. In addition, levels of other inflammatory mediators such as CRP have not been investigated. Therefore, the reviewer could not understand if IL-6 is the specific inflammatory molecule that is related to the level of sFe, or if IL-6 is one of the inflammatory molecules related to the level of sFe. If the latter is true, the low-grade systemic inflammation, not only IL-6, contributes to lowering the levels of sFe. Without the information on other inflammatory molecules, the reviewer could not assess if IL-6 is a major contributor for the sFe levels, or if IL-6 is merely a biomarker for sFe level.

Response: We appreciate the comments from the reviewer. As the reviewer pointed out, IL-6 may only be one of the inflammatory molecules related to the level of serum iron. Production of CRP is enhanced by IL-6. Therefore, assessment of other inflammatory markers, including high-sensitivity CRP, would be informative. We have added this information to the Discussion as a limitation of the present study (page 15, line 16 to page 16, line 2).

2. Recently, Shibata and colleagues demonstrated the relationship between sFe levels and spirometric parameters, namely FEV1. Because FEV1 is largely affected by the behavior of cigarette smoking, the effects of cigarette smoking on the sFe levels may not be ignored. The reviewer thinks that it is better to include cigarette smoking behavior as a covariate in the multiple linear regression analysis.
Response: We agree with the reviewer that the behavior of cigarette smoking is important. However, because only a few participants were current smokers in this study, unfortunately, we could not assess this issue.

Finally, we have checked and amended English expression throughout the manuscript again.