Author’s response to reviews

Title: Do Pregnancies Reduce Iron Overload in HFE Hemochromatosis Women? Results from an Observational Prospective Study

Authors:

Virginie Scotet (virginie.scotet@inserm.fr)
Philippe Saliou (philippe.saliou@chu-brest.fr)
Marianne Uguen (virginie.scotet@gmail.com)
Marie-Christine Mérour (marie-christine.merour@efs.sante.fr)
Céline Tripogney (celine.triponey@efs.sante.fr)
Brigitte Chanu (brigitte.chanu@efs.sante.fr)
Jean-Baptiste Nousbaum (jean-baptiste.nousbaum@chu-brest.fr)
Gérald Le Gac (gerald.legac@chu-brest.fr)
Claude Férec (claude.ferec@univ-brest.fr)

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Author’s response to reviews:

Dear Editor,

Please find enclosed a revised version of our manuscript entitled “Do Pregnancies Reduce Iron Overload in HFE Hemochromatosis Women? Results from an Observational Prospective Study” and referenced PRCH-D-16-00615, as well as our point-by-point responses to the reviewers.

We have been able to take into account all the remarks made by the reviewers.

Hoping that our manuscript will be accepted for publication.
Sincerely yours,

Claude Férec & Virginie Scotet

Please note that the page numbers mentioned in the following responses are those of the revised version with marked changes.

REVIEWER 1: Kyly Whitfield

Overall

Throughout this paper you state that this is an epidemiological study. However, with your convenience sampling, and lack of power calculations and wavering primary outcome measures I wonder if this study would be better termed an exploratory analysis.

I wonder if the study was underpowered for the primary outcome measure. Please provide more details on what the primary outcome of the study is, and how this sample size was determined.

I am concerned that a major piece of this paper surrounds the sex analysis of age of diagnosis, yet not information is provided about the men included in this analysis. This needs to be included in the study design, methods, and ethics sections.

The main point of this paper is to assess whether pregnancy has an effect on iron accumulation in women with HFE hemochromatosis, yet so little is known about blood losses around women’s pregnancies, as well as other iron-related information (perinatal iron supplementation, postpartum amenorrhea, etc). Also, were fetal losses included? Why or why not?

Statistical methods a bit fuzzy at this moment – please go through and clarify methods, and also ensure that all stats included in the paper are documented in the statistical analysis section.
This paper is very interesting and I think with re-working will be ready for publication.

We are very grateful to the reviewer for all her remarks, which made it possible to improve the manuscript. We have responded to all of these remarks in the points below.

Abstract

pg 2 line 7: ‘recessive’

We have corrected the misspelling. We have changed “recessibe” by “recessive”.

pg 2 line 42-49: The conclusions of this abstract focus mostly on a completely different study – the mouse model study and others’ research on hepcidin, which was not assessed here at all. Please re-work Conclusions section to reflect only the results of this study without speculation.

As requested by the reviewer, we have modified the Conclusions section of the abstract. We have added one sentence to better reflect the results of the study and we have shortened the last sentence.

Text

pg. 3 line 5: ‘inborn’ rather than ‘innate’

This change has been done.

pg. 3 line 14: It is important to note that humans do not have the ability to regulate iron

To be clearer, we have mentioned this in the text (Background – § 1 – p. 3).

pg. 3 line 16: re-wording
We have re-worded the corresponding sentence (Background – § 1 – p. 3). We have changed “... and leads, if not treated early, to serious clinical complications including cirrhosis, carcinoma and cardiomyopathy” by “... and may result in serious damages (such as cirrhosis, hepatocellular carcinoma or cardiomyopathy)”.

pg. 3 line 31 and line 52: sex, not gender
This change has been done.

pg. 3 line 47: Could the authors please elaborate here? It is wild that this gap in the research still remains. Could the authors detail and cite other related studies that have investigated similar outcomes? And/or authors should cite the current dogma as to why pregnancy is a biologically plausible reason for positively impacting the health of women with hemochromatosis.

As we mentioned in the Discussion section (§ 2 – p. 12), “no epidemiological study has so far specifically investigated the relationship between pregnancies and iron burden in HFE hemochromatosis in humans”. Some studies have nevertheless shown interest to pregnancies [Moirand et al. Ann Intern Med 1997; Deugnier et al. Br J Haematol 2002; Desgrippes et al. Hepatology 2013] and these related studies were detailed in the Discussion section (§ 2 – p. 12). As requested by the reviewer, we have added references that cite the dogma as to why pregnancy is a biologically plausible reason for impacting the health of women with HFE hemochromatosis (Background – § 4 – p. 3).

pg. 4 line 21: Details of this Ethical Review Board (both board and approval number) should be stated here. Also state here that women provided written informed consent. I see this detailed on pg. 13 – I think this should also be stated in-text.

Details of this Ethical Review Board were mentioned in the § “Declarations” at the end of the manuscript. As suggested by the reviewer, we have put this paragraph in the Methods section (§ Study design and participants – § 3 – p. 5) and have added the approval number.

As explained, this study was conducted in accordance with the principles expressed in the 1975 Declaration of Helsinki. The study protocol was approved by the Medical Ethical Committee of the Hospital University of Brest on December 15th, 2003 (n# 2003_15-12_EMI 0115). Written informed consent was obtained from each participant.

pg. 4 line 19: remove “the achievement of”
This change has been done.

pg. 4 line 33: delete ‘yielded notably’; replace with ‘collected’
This change has been done.

pg. 5 line 17: again, sex not gender
This change has been done.

pg. 5 line 21: If men were also included in analysis, their recruitment, consent, etc must be detailed, and their demographics should also be shown. Please confirm that men also provided written, informed consent for their data to be used in this study.

The present study focuses on pregnancies (and thus on women), but our research programme on HFE hemochromatosis relies both on men and women that have been enrolled in a phlebotomy programme in the blood centre of Brest during the study period. Therefore, men also provided written informed consent to participate in the research protocol.

In the present study, data on men were used only to confirm whether women were diagnosed later than men. For this reason, we do not think it is necessary to present, in this paper, the demographics of men.

The characteristics of men included in our research programme on HFE haemochromatosis have already been described in detail in a previous paper (Saliou et al. PloS One 2013). We have added this reference in the Methods section (§ 1 – p. 5).

pg. 5 lines 14-27: Were demographic statistics computed? Describe here.
We have described the socio-demographic characteristics of women and compared them according to the number of pregnancies.
As requested by the reviewer, we have better described the statistical tests used and we have ensured that all the tests included in the paper were documented in the Statistical analysis section. We have added the following paragraph in the Methods section (Statistical analysis – § 2 – p. 6): “Continuous variables were described using mean and standard deviation (SD), and compared using Student’s t test or Anova. When these variables were not normally distributed, they were described by median and interquartile range (IQR), and compared by the Mann-Whitney test. Categorical variables were summarised using percentages, and compared using $\chi^2$ test or Fisher’s exact test when appropriate.” Moreover, we have inserted a superscript under the tables to describe the statistical tests employed.

Since our study involves several stages (study of the evolution of the sex ratio, study of the influence of pregnancies on serum parameters), we think it was more understandable to conduct the analysis as we did.

Regarding study power, the sample size calculation had been done with the serum parameter that best reflects iron overload, i.e. the amount of iron removed by phlebotomies. For that calculation, we had assumed a difference of 1 gram of iron removed between the group of women who had pregnancies and the group of women who had not (2.5 g versus 1.5 g) and the number of subjects required had been estimated at 13 per group, by considering a $\alpha$ risk of 5% and a study power of 80%. Our study appears therefore powerful.

It should be noted that our study is exhaustive over the study period (2004-2011) because it has included prospectively all the C282Y homozygous hemochromatosis women enrolled in a phlebotomy programme at our blood centre over that period.

What statistical inference test was employed to compare these three groups? ANOVA? Please describe in Methods section.
In table 3, categorical variables (percentages) were compared using a test \( \chi^2 \) test whereas in table 4, continuous variables (that have all a non normal distribution) were described by median and interquartile range and compared using the non parametric Mann-Whitney test. As mentioned above, all the tests used have now been described in the Methods section (Statistical analysis – § 2 – p. 6).

pg. 7 line 48 and lines 53-56 and pg. 8: If these results are not significant, then you cannot actually say that the SF/AIR concentrations were higher. Maybe you were underpowered for these analyses? Remove all of these statements, please.

As requested by the reviewer, we have removed all of the corresponding statements and have reworded the Results section (§ Influence of pregnancies on iron markers – p. 9-10).

pg. 9 line 14: sex, not gender
This change has been done.

pg. 9 line 44: Please cite this statement – why is excessive alcohol intake a risk factor?
Alcohol is likely to increase serum ferritin by a direct mechanism of induction of its synthesis and by two indirect mechanisms, one by cellular toxicity (cytolysis) and the other one by decreasing the production of hepcidin, the key regulator of iron homeostasis. Excessive alcohol intake is, with metabolic syndrome, one of the main secondary causes of hyperferritinaemia. We have mentioned this in the Discussion section (§ 3 – p. 11) and have added one reference [Deugnier et al. Gastroenterol Clin Biol 2009]. We have also explained the influence of excessive alcohol consumption on HFE hemochromatosis in the Method section (§ 1 – p. 7) and have added two references on this topic [Fletcher et al. Gastroenterol 2002; Scotet et al. AJE 2003].

pg. 9 line 56: You also did not have data on blood loss from labor and delivery, the major contributor of iron removal due to pregnancy. Note that maternal breastfeeding duration would have little effect on iron status of the mother as very little iron is transferred to the milk. You note that you are missing vital data around return to menses post-partum (lengthier amenorrhea would be associated with excess iron) and although 10 weeks is the average in France (which you need to cite there), you should indicate this as more of a limitation of this research.
The reviewer is absolutely right. Unfortunately, we do not have information on blood loss from labor and delivery. Therefore, we have added these factors in the list of potential confounders presented in the Discussion section (§ 4 – p. 11).

Regarding maternal breastfeeding and return to menses, we have modified a sentence in the Discussion section (§ 1 – p. 12) to include the remarks of the reviewer. We have written that “Some of them may also only have a small impact on iron status, as maternal breastfeeding because its duration (with its consecutive amenorrhea) is in average relatively short and because very little iron is transferred to the milk”.

pg. 10 lines 42-44: This seems too low. The Institute of Medicine recommends iron intake of 18 mg/d preconception, which increases to 27 mg/d during pregnancy. Please double check on this.

We have re-read the concerned references (and added some new ones) and all mention the figures quoted. The doses recommended by the Institute of Medicine correspond to the amounts of iron to be ingested to cover the increase in iron requirements. The data presented in the paper correspond to the increase in daily requirements for absorbed iron. We have specified this in the text (Discussion – § 4 – p. 12).

pg. 10 line 33: Cite.

We have added a reference from the French Institute for Demographic Studies, who reassessed the fertility rate in France in 2016. This rate was lower than in 2010 (1.93 per women). We have made the corresponding change in the text (Discussion – § 3 – p. 12).

pg. 10 line 52: Cite.

We have added the corresponding references (Discussion – § 1 – p. 13).

pg. 11 lines 37 onwards: sex, not gender.

This change has been done.
pg. 12 line 15: As currently phrased it sounds like you’re calling your study not dedicated! “Our work challenges an old and well-established, yet unproven, hypothesis that pregnancy slows iron accumulation in women with HFE hemochromatosis.”

The reviewer is totally right. We have changed our sentence by that she proposed (Conclusions – p. 14).

Table 1

- Why was the age cut-point set at 60 y? Provide reasoning, or remove and just state mean (SD)

The cut-point was set at 60 years because it is the classic cut-point found in the literature for the beginning of the expressivity of the disease in women. We have mentioned this under all the concerned tables.

- Provide superscript and insert reference to ‘excessive’ alcohol intake, citing WHO

The definition of excessive alcohol intake is provided in the Method section (Statistical analysis – § 2 – p.8). We have also added it under the table.

- What was the mean (SD) age at menopause onset?

Since Table 1 and Table 3 have been merged and that the merged table is now more consistent, we have retained only the description of the qualitative variables in this table. Therefore, the description of age at menopause is now presented only in the text (Results – § 1 – p. 8).

- Chronic bleedings – this needs to be defined. Is this medically indicated phlebotomies?

Chronic bleeding includes gastrointestinal bleedings, chronic hematuria, bleedings due to parasitic infections. This has been specified in the Methods section (Questionnaire – § 2 – p. 5-6).

Table 2

- Title confusing – please re-word. A better title might read “Age of HFE Hemochromatosis Diagnosis stratified by sex”
We agree that the title was confusing. We have re-worded it. However, table 2 does not present the age at diagnosis stratified by sex, but it presents the distribution of men and women by age at diagnosis.

- Again, where are the demographics on the men included in this analysis? If they are included in the data in Table 1, then they should also be described in Table 1.

As mentioned above, data on men were used only to confirm whether women were diagnosed later than men. For this reason, we do not think it necessary to present, in this paper, a detailed description of the characteristics of men.

- What statistical test was used to compare the mean age of diagnosis between men and women (also in Abstract Methods)

As mentioned in the Methods section (Statistical analysis – § 3 – p. 7), we used a Student’s t test to compare the mean age at diagnosis between men and women.

Table 3

- This is the same information presented in Table 1; merge these two tables by adding a column directly to the right of the “Variables” column entitled “All Women”.

As suggested by the reviewer, we have merged tables 1 and 3 and changed the number of the following tables in the text. As table 1 relied on all the women (n=140) whereas table 3 relied on the women for whom data on pregnancies was available (n=137), we have made some changes in the first paragraph of the Results section (p. 8) to take this into account.

- Insert a superscript to describe the statistical test employed to make comparisons between the three groups.

This has been done.

- Include all comments made for Table 1:

All these comments have been included in the merged table.
Table 4

- The title of this table indicates that these parameters were measured before the phlebotomy programme commenced, so where are there values for “amount of iron removed”?

Transferrin saturation, serum ferritin and haemoglobin were measured at entry in the phlebotomy programme whereas the amount of iron removed by phlebotomies was measured at depletion. The title was confusing and we have re-worded it to be clearer. We wanted to say that table (now table 3) presents the biological parameters according to the number of pregnancies that women had prior to entry into the phlebotomy programme.

Tables 5 & 6

How were these variables chosen to be fit into this model? For example, what is the biological plausibility between alcohol intake and excess iron? In the Methods section, provide more information on how these predictor variables were selected, and how the linear models were built.

We have better explained the choice of the variables introduced into the model in the Methods section. As mentioned above, we have notably explained the biological plausibility between alcohol intake and excess iron (§ 1 – p. 7) and have added references on this topic.

We have also provided more information on how the linear models were built and how the regression coefficients were interpreted with logarithm transformation of the dependant variable (Statistical analysis – § 1 – p. 7).

REVIEWER 2: Eva K. Pressman

This is an interesting and important study looking at the effect of prior pregnancies on the long term outcomes and need for iron removal in patient with hemochromatosis. The study is well done overall with comparisons to men and comparisons by number of pregnancies but does not include any information on the actual pregnancies.

Were the pregnancies all carried to term or did they include preterm deliveries, miscarriages or abortions? Were any of them complicated by postpartum hemorrhage or other complications that could affect iron stores (pre-eclampsia, abruption, placenta previa). Without these pregnancy details, it is more challenging to understand this data. At the very least, the manuscript should discuss if miscarriages or abortions were included in the pregnancy numbers. Pregnancies that end in the 1st trimester are much less likely to affect iron stores.
These remarks are very relevant. The pregnancies considered in the present study did not included miscarriages or abortions that occurred in the first trimester and that are much less likely to affect iron stores. We have realized that this was not mentioned in the text and so we have added it in the Methods section (§ Questionnaire – (§ 2 – p. 5). Unfortunately, we do not have information on postpartum hemorrhage or pregnancy complications that could affect iron stores, but such events remain relatively rare. We have added these factors in the list of potential confounders presented in the Discussion section (§ 4 – p. 11).

REVIEWER 3: Elvira Guerra-Shinohara

This is an interesting study, but some questions need to be clarified.

1- The numbers of individuals in the tables vary and there are no answers. What is the impact of these missing information?

We have indeed missing values at some variables, but this concerned less than 3% of the patients in mean. Moreover, as we mentioned in the Discussion (§ 2 – p. 11), “we also ensured that the baseline characteristics of women excluded from the multivariable analysis (due to missing values) did not differ from those of included women”, and therefore that missing values did not cause bias.

2- How long ago occurred births, regular blood donations, chronic bleeding or blood transfusions? This time can not be a confounding factor?

We agree that time should be a confounding factor. Patients were asked, at entrance in the phlebotomy programme, if they were regular blood donors, if they had chronic bleedings and if they received blood transfusions. Unfortunately, we do not have information on how long ago these factors occurred, nor how long exactly they lasted. We only know if they were present or not. Therefore, we took them into account through dichotomous variables and did not observe any effect. For pregnancies (our main explanatory variable), we have mentioned in the Discussion section (§ 1 – p. 12) that “we were aware that it would have been ideal to know the delay between various pregnancies, as well as between the last pregnancy and the beginning of the treatment”.
3- Table 4 - it is not clear in the table what is IQR.

IQR means interquartile range, what corresponds to the range between the first and the third quartiles. It is often written as [Quartile 1 – Quartile 3]. The meaning of this abbreviation was indicated in the title of table 4, but in order to be clearer, we have put it under the table. Its meaning was also added in the Methods section (Statistical analysis – § 2 – p. 6).

4- Usually, the value p is described to three decimal places.

As suggested by the reviewer, we have removed one decimal from all the p-values.