Author's response to reviews

Title: A new 500kb haplotype associated with high CD8+ T-lymphocyte numbers predicts a less severe expression of hereditary hemochromatosis

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Author's response to reviews: see over
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Dear Editor of the BMC-Medical Genetics,

Please find enclosed the manuscript re-revised “MS: 8672449692115360 - A new 500kb haplotype associated with high CD8+ T-lymphocyte numbers predicts a less severe expression of hereditary hemochromatosis” which my colleagues and I changed accordingly to the reviewers’ comments.

We would like to thank for the careful revision of the manuscript. The detailed point-by-point response to reviewers’ is indicated below (in blue):

Reviewer: Ana Martinelli
Reviewer's report:
The manuscript fulfills all the criteria to be accepted for publication as it is.
No changes are needed

Reviewer: Lawrie Powell
Reviewer's report:
Major Compulsory Revisions
I have no major compulsory revisions.

Minor Essential Revisions
I have no minor essential revisions and I think the paper warrants publication in its present form.

Discretionary Revisions
No comment here.
No changes are needed
Reviewer: Jill Waalen  
Reviewer's report: 
Minor Essential revisions
1. p. 14, para 2: The p values given for association of the SNP markers and total lymphocyte counts are exactly the same as the p-values reported in the paragraph above it for the association with CD8+ counts. This appears to be an error. 
There was indeed an error in the p values for association of the SNP markers and total lymphocyte counts that was corrected.

2. p. 14, para 2: The mean total lymphocyte counts and SD associated with each SNP are exactly the same (i.e. 2.00 ±0.58), which also seems to be an error. 
The mean total lymphocyte counts and SD associated with each SNP is very similar (2.00±0.58 and 2.01±0.58).

Discretionary
1. p. 5, para 1, last sentence: “namely new genetic modifiers” should be changed to “including new genetic modifiers” as other modifiers of phenotypic expression, including environmental and epigenetic factors, are also being considered. 
Changed accordingly.

2. p. 5, para 2, sentence 2: “as reflect of” should be “reflective of” 
Changed accordingly.

3. p. 5, para 2, sentence 4: “the setup of a stable number of peripheral CD8+ T lymphocytes … “ should be changed to “stable numbers of peripheral CD8+ T lymphocytes” 
Changed accordingly.

4. p. 9, para 2: It is stated that the median value was used as a cut-off to define “high” and “low” lymphocyte numbers. This is a bit unconventional – is there a “normal” range? -- and the results related to these definitions were not included in the Results section, but rather the Discussion. I would eliminate this issue from the Methods and Discussion. 
We used the same criteria to classify in “low” or “high” as used in our previous papers on this subject (refs 21, 22, 26). The cut-off values were based on the median values of
each parameter and established on a control population of 265 healthy subjects from north of Portugal (ref 21). The paragraph was changed.

5. p. 17, para 4: This section is titled “Global impact …” Given these results their own section with this title seems to give them more importance than they are due. This is simply a pooled analysis and could be introduced: “When results from both populations were pooled, the results were similar”. Changed accordingly.
Reviewer: K Sigvard Olsson
Reviewer's report:

1. In the introduction the authors report that one additional study (ref 20) has supported their previous findings indicating an inverse relationship between lymphocyte counts and severity of iron loading. However, it should be noted that this relationship was significant only for those who had inherited a certain haplotype (HLA-A1-B8) and also that those with the most severe phenotype = cirrhosis, in this larger patient series, had lymphocyte counts not different from those without cirrhosis.

The purpose of reporting this study was to refer the only study in “non-Portuguese” subjects where lymphocytes were associated with clinical expression. We are aware that this association was linked to the HLA A1-B8 haplotype but, however, the discussion about the link to HLA is out the scope of this paper therefore it was not reported.

2. Study population:
From their previously reported patient series of 64 adult hemochromatosis patients of whom 22 were detected at family investigation (ref 26) they have now selected 56 patients, 45 probands and 11 family members for their study.

The reason why in the present study we have 11 family members and in a previous study (ref 26) we had 22 is because some of the patients were not included in the SNP analysis for the following reasons: a) in 5 patients we didn't have available DNA; b) 5 patients had only one determination of CD8+ T-lymphocyte counts and subpopulations and could not be confirmed; c) there was one patient that had history of Hepatitis B and highly variable numbers of CD8+ T cell counts along time.

They have also included 10 homozygous HH patients from Vancouver, Canada, because of “different ethnicity”, ignoring the view that they most probably are descendants to the same common north European ancestor as their own patients from Portugal.

We agree with this comment. We have included in Discussion a comment with the view that Canadian patients may have the same common ancestor of Portuguese patients (Discussion, page 19, #2, starting on line 10).
3. Clinical characterization of subjects
The patients were divided in two groups according to the presence or absence of symptoms.
From the experience gained in the above mentioned studies (ref 10,11), this is hardly recommendable. A liver affection was the only symptom or sign that was more common in hemochromatosis subjects than in controls. What the authors wanted to study is the influence of genetic factors upon iron loading, not the development of symptoms! The authors are well familiar with previous studies where a cut off value for a major iron overload has been set at serum ferritin of $1000 \mu g/L$ or 5 grams of total body iron stores (TBIS) (ref 9). This is because a severe liver damage (fibrosis, cirrhosis) is a rare complication below this cut off.

For the purpose of analysis, patients were never grouped according to the presence or absence of hemochromatosis related symptoms. On the contrary, they were grouped according to genotype (Results-Section 2) and the percentage of symptomatic patients estimated in each group. Perhaps some confusion was introduced in Material and Methods where the clinical characterization of patients was presented. This paragraph in Material and Methods was changed to be clearer.

In the result section page 18 the authors report that all patients with severe iron overload $>5$ g are homozygous for AAT. Why not use such a cut off value or another where the influence of age is considered?
For the same reason as above we did not divide patients in groups according to degree of iron overload (TBIS). We used TBIS as a continuous variable to be estimated in the groups of patients divided according to genotype (Table 2).

Page 9 middle (95% confidence interval) check the figure 0.419!
The 95% CI presented is for the mean. We completely agree with the referee that this figure is confusing. According to the comments of another referee we excluded this paragraph and referred to our previous papers on hemochromatosis to define the cut-off values used.

4. Results
Table 1 shows data of 56 subjects, table 2 only 52?
Three patients were not included in this table because 2 were hepatitis B carriers and one had chronic monoclonal expansion of CD8+ T cells (this is now referred in Material Methods-Statistical analysis), and a fourth patient (patient with the haplotype #14-Table 1) was not included because he doesn’t have either the AATxAAT haplotype or the GGGxAAT. This is now referred in Table 2 (footnote).

5. Discussion
We agree with the very pertinent comments of the referee about the need to discuss the issue of which factor is the modifier. We extended the discussion to further stress two points: first the possibility that the patients with the new haplotype (G-G-G) may represent the tip of an iceberg and this may eventually be a major common haplotype in worldwide asymptomatic populations of hemochromatosis patients and secondly the fact that the major limitation of this study is the lack of a large number of asymptomatic patients (naturally they do not appear in the clinical practice) and the need to extend the study to include more mildly or asymptomatic patients.
Hoping to have the paper accepted after having answered to the referees’ comments.

Sincerely Yours,

Eugénia Cruz.