Author's response to reviews

Title: HLA HAPLOTYPES ASSOCIATED WITH HEMOCHROMATOSIS MUTATIONS IN THE SPANISH POPULATION

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

enclosed please find the new version of the paper entitled: “HLA HAPLOTYPES ASSOCIATED TO THE HEMOCHROMATOSIS MUTATIONS IN THE SPANISH POPULATION” by Pacho et al. (Manuscript ID 1402390333713256).

Thank you for your help,
Best regards

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POINT-BY-POINT DESCRIPTION OF THE CHANGES MADE

Answers to reviewer 1 (Ronald Acton)

Question
HFE should be in italics.

Answer
It has been done.
**Question**
The nomenclature for HLA alleles is not correct. Example HLA-A3 should be HLA-A*03, HLA-B44 should be B*44. See: Bodmer, JG Vox sang 1999;77(3): 164.

**Answer**
In this paper we identified the HLA specificities with serologic typing. The WHO Nomenclature Committee for Factors of the HLA System advises to use HLA-A3 when the allele is serologically defined, and HLA-A*03 when defined at the DNA level by a generic typing approach. See Schreuder, Human Immunology 60, 1157-1181 (1999).

**Question**
In the background section, first paragraph line 10: provide references for the frequency of HFE mutations that are found in various populations.

**Answer**
It has been done.

**Question**
Provide reference for odds ratio.

**Answer**
It has been done.

**Question**
In the results section table 3 is presented before Table 2. This is confusing present Tables in order that data is discussed.

**Answer**
It has been done.

**Question**
Although the authors refer to the Barton paper as the only other paper where HLA haplotypes are presented they fail to make a major distinction between the paper by Barton and Acton and their own. The major difference is that the paper by Barton and Acton presents haplotype frequencies assessed by family studies where phase could be set, whereas their paper estimates haplotype frequencies. Haplotype frequencies that are estimated are not as accurate as those determined by family studies. This difference in the two studies should be noted in the discussion.

**Answer**
After the first paragraph in the discussion section, the following paragraph has been added:“The paper by Barton and Acton [19] presents haplotype frequencies assessed by family studies where phase could be set; in our paper, the haplotype frequencies are estimated because the probands and controls are unrelated individuals”.


Question
In the discussion under the section C282Y and H63D mutations: which one is older, second paragraph, line 12 reference is made to “haplotype has broken”. I think what the authors mean is that there has been more recombination in the HLA-A29/B44/H63D haplotype than the HLA-A3/B7/C282Y haplotype.

Answer
“haplotype has broken” has been changed by “haplotype has suffered more recombinations”.

Question
In Tables 1, 2 and 3 odds ratio appears twice as a footnote.

Answer
It has been corrected.

Answers to reviewer 2 (M. D. de Juan)

Question
In methods, the last phrase should say: ..were detected using DNA-based and microlymphocytotoxicity…techniques respectively

Answer
In Abstract (methods, page 1, line 24): “techniques respectively” has been added.

METHODS:
1. In this section the authors should give any data about the characteristics of the C282Y and H63D homocygotes individuals. Are they hemochromatos is patients or simply individuals with iron metabolism alterations or healthy people?

Answer
In the first paragraph of METHODS (page 2, line 43), the following phrase has been added: “Probands used were selected by a presumptive diagnosis of hemochromatosis using an elevated transferrin saturation criterion and medical care.”

Question
And the group of controls, what do you mean with "normal", you should explain this in order to better differentiate both group of individuals

Answer
First paragraph of METHODS (page 2, line 42). “normal subjects” has been changed by “apparently normal subjects”

Question
2. A head title after HLA-A, typing , in order to separate the rest of the methods saying: HFE
Question

RESULTS:
1. The analysis of HLA frequencies in individuals carrying C282Y and H63D in homocgyosity is really an indirect way to study linkage disequilibrium between HLA-A and B antigens and HFE mutations. Strictly it should be done by comparing these HLA frequencies in C282Y or H63D homocgyotes versus a group of controls without HFE mutations. Have you studied this control population for HFE mutations?. If the answer is no, you should comment this idea in Results section. The significative associations found in your analysis between HLA-A3 and C282Y and A29-H63D probably would not change if you use a control group without HFE mutations but you should comment this. For example, H63D mutation is very frequent in mediterranean general populations and could introduce a bias in your results.

Answer

After last paragraph of the RESULT section (page 4, line 11), the following phrase has been added: “The significant associations did not change if we use a control group of 230 individuals without HFE mutations”.

Question

2. With only 17 individuals heterozygous for the S65C and no information about HFE mutation state of the control group you should say in the discussion that "we find that S65C mutation seems to be linked to HLA-A26" And also separate this argument as a secondary finding in the conclusions section.

Answer

In the DISCUSSION section, page 5, line 46, “is linked” has been changed by “seems to be linked”.

Question

3. There is probably a mistake in Table 3: Is OR= 32.7 for A29/B14 and A29/B62 haplotypes?????

Answer

The fact is correct

Question

4. In table 3, It woul be easier to read if you keep the same heading design used in tables 2 and 3(including the term Freq; H63D or C282Y haplotypes instead of Chromosomes..etc).

Answer

The table 3 has been changed according the suggestion of the reviewer.
Question

DISCUSSION:
1. H63D and HLA: As far as I understand your results don’t agree with those referenced in (2) (Porto et al) but the results in this manuscript do agree with results showed in (19) (Cardoso et al). They find LD between H63D and several HLA-A29 containing haplotypes both in Hemocromatosis families and in general population. This finding is confirmed with success in this paper with homozygous H63D and C282Y individuals.

Answer

The phrase “but not in linkage disequilibrium with either A29 or the H63D mutation” has been deleted. In addition, “On the contrary” has been deleted.

Answers to reviewer 3 (Graca Porto)

Question

a) For allele associations.

To perform their study, the authors have selected a large population of individuals homozygous for the mutations (except for S65C given the low frequency of this mutation). In the Methods section, the description of individuals should be more complete. One may assume that the 100 unrelated individuals homozygous for the C282Y mutation are hemochromatosis patients but nothing is referred about the clinical status of the H63D homozygous, who are not included in the “unrelated normal subjects”. Are they patients with iron overload? Are they taken from the normal population? Who are the controls? Were they HFE genotyped?

Answer

- In the first paragraph of METHODS (page 2, line 43), the following phrase has been added: “Probands used were selected by a presumptive diagnosis of hemochromatosis using an elevated transferrin saturation criterion and medical care.”
- First paragraph of METHODS (page 2, line 42). “normal subjects” has been changed by “apparently normal subjects”
  - 230 individuals from the control group were also HFE genotyped.

Question

b) For haplotype associations

Another question in the Methods section is about the haplotype assignment. How were haplotypes defined? There is no reference to family segregation studies. This is quite relevant because if haplotypes are defined not by family segregation but on the basis of the assumed linkage disequilibria, there may be an important bias towards the results because we are facing a round argument, i.e., we conclude that there is a linkage to the haplotype that we “a priori” assume that is in linkage disequilibrium. Of course it is very plausible that the A29B44 is the ancestral haplotype but the fact is that, in terms of haplotype frequencies, in a previous study where H63D carrying haplotypes were defined in the Portuguese population on the basis of family segregation (reference 19 in the text) there were more H63D-A29 carrying haplotypes without B44 (60%) than H63D-A29-B44 haplotypes (40%), in contrast with the results presented here in a Spanish population where 65% of all H63D-A29 carrying haplotypes are also defined as B44. This point should be clarified and discussed if, indeed the haplotypes were assigned by linkage disequilibrium.
Answer
- The following paragraph has been added to the Statistical analysis section: “The haplotype frequencies were computed using the Expectation-Maximization algorithm [16]; this procedure is a interactive process aiming at obtaining maximum-likelihood estimates of haplotype frequencies from multi-locus genotype data when the gametic phase is unknown.”
- In table 3 we can see that the P value of the H63D-A29 association (<10^-7) is smaller than the H63D-B44 association P value (2.10^-5). We think this agrees the results obtained in reference 19.

Question
Discussion, H63D and HLA, second paragraph, line 3: do the authors really mean Danish population or Australian? (ref.18).

Answer
This was a mistake. The reference has been changed by “Milman N, Graudal N, Nielsen LS, Fender K: An HLA study in 74 Danish hemochromatosis patients and in 21 of their families. Clin Genet 1992; 41: 6-11”.

Question

Answer

Final consideration
The English language has been checked by a native speaker.