Author's response to reviews

Title: Two common nonsynonymous paraoxonase 1 (PON1) polymorphisms are not related with the risk for brain astrocytoma and meningioma

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Author's response to reviews: see over
Sirs,

Thank you for your letter and for the opportunity to resubmit the manuscript MS: 2011529362335605 entitled “Paraoxonase 1 (PON1) polymorphisms are not related with the risk for brain astrocytoma and meningioma”.

We are thankful to reviewers and we agree with their comments. The manuscript was extensively revised according their suggestions and all concerns raised by the reviewers are addressed in the revised version of the manuscript. Changes are in red font in the revised version of the manuscript.

Reviewer Comments:

**Reviewer #1:**

*Reviewer's report:*

1) The question posed by the authors is not well defined. The authors presumably try to associate slow metabolism of organophosphorous compounds by polymorphic forms of PON1 with brain tumors. However, this link is rather weak and cannot be generalized for all of the currently used organophosphates (OPs). There are several OPs such as chlorpyrifos, diazinon, fenitrothion and primiphos-methyl. These compounds are substrates for PON1 and there are polymorphisms in PON1 gene that affect enzymatic activity. Although not clearly put forward, the hypothesis implied by the authors makes sense until here. However, the problem is, rate of metabolism of organophosphorous pesticides by a given PON1 isoform, thus the extent of protection against a given OP changes with the specific compound.

In a study carried out by Li et al. 2000 (Li et al. Pharmacogenetics 2000;10:1-13), either human PON1 192Q or 192R isoform was injected intravenously into PON1 knockout mice and the effects of OPs on brain and diaphragm AChE were determined. In the case of chlorpyrifos oxon, both isoforms were protective, and PON1 192R offered about 50% better protection than PON1 192Q. Both PON1 192R and 192Q offered equal protection against diazoxon, and neither human PON1 isoform protected against the toxicity of paraoxon, extending the surprising findings described above. The results from the kinetic analysis of substrate hydrolysis by purified human PON1192 isoforms provide an explanation for such
findings. Although the PON1R192 isoform is eight times more efficient than the PON1Q192 isoform in hydrolyzing paraoxon (Km/Vm D 6.27 versus Km/Vm D 0.71), neither isoform hydrolyzes paraoxon as efficiently as diazoxon or chlorpyrifos oxon (Li et al. 2000). The hypothesis would be alright if a given PON1 isoform protected against all of the currently used OPs to the same extent or only one OP compound was in use. Thus, it is difficult to determine which PON1 isoform represents a risk factor for the development of brain tumors. The authors did not mention which isoform is expected to be the risky one and why. They did not discuss the differential activity of PON1 genotypes towards different OP substrates.

Thank you for the observation. We strongly agree. A new paragraph addressing this point has been included in the discussion section.

2) Authors have determined only the genetic polymorphisms of the subjects and did not measure PON1 activities and/or concentrations. However, genetic polymorphisms are not the sole determinants of PON1 activity; it was reported that paraoxonase activities can vary by 13 fold within a given genotype (Jarvik et al. Arterioscler. Thromb. Vasc. Biol. 2000;20;2441-2447; Richter R, Furlong CE. Pharmacogenetics. 1999;9:745–753; Brophy et al. Pharmacogenetics.2000;10:453–460). Several factors affect PON1 activity, such as physiological and pathological states, dietary and lifestyle factors and environmental chemicals. In the study of Jarvik et al 2000, no genotype effect was detectable unless activity phenotype was also considered. Therefore, it was strongly suggested that PON1 activities and/or concentration should be determined together with PON1 genotypes for correlation with disease susceptibility (Mackness et al. Arterioscler Thromb Vasc Biol 2001;21(9):1451–7; Draganov et al. Naunyn-Schmiedeberg’s Arch Pharmacol 2004;369(1):78–88).

The authors state in the discussion that: “PON1 should hypothetically act as a detoxifying enzyme at this level, causing the hydrolysis of the acetylcholinesterase-inhibiting oxons (activated intermediates) of some organophosphorus compounds [29], decreasing the possible arrive of these compounds to the brain”. Studying only the genetic polymorphisms of PON1 is not enough to reject this hypothesis. Even the authors themselves touched this point in the discussion, when they cited the study by Kafadar et al. who found decreased PON1 activity in brain tumor patients compared to controls, but couldn’t find an association between PON1 192Q/R genotypes and brain tumors.

Measuring PON1 activity of patients and controls towards several OP substrates would give valuable tools to determine subjects susceptible to OP poisoning. Then, an association between brain tumors and susceptibility to OP compounds could be sought.

Authors should have discussed the reasons for not employing PON1 activity and/or concentration determinations and make it clear that they are already aware of the limitations of such a genetic association study.
The reason for not measuring PON1 activity is that only DNA samples, but not whole blood, were available from patients. This is a limitation of the study and a sentence indicating that we are aware of this limitation was included in a new paragraph in the discussion section.

3) No information is given on any incidence of exposure of the patients and controls and/or their parents to organophosphorous compounds. As discussed by the authors, in the study by Searles-Nielsen et al. there was a significant association between PON1 -108T allele and exposure to pesticides.

Recruiting brain tumor patients from agricultural workers or people who claim to be exposed to pesticides would have been more informative as to the role of PON1 in formation of brain tumors.

We agree. Unfortunately these patients were recruited before association of PON1 alleles and exposure to pesticides was described. A sentence indicating when patients were recruited was included in the Methods section.

4) In the abstract, it is not put forward clearly why the authors decided to study PON1 polymorphisms in brain tumors. In discussion section, the background information supplying the link between xenobiotics (including OPs, environmental pollutants and drugs) and brain tumors is missing.

A new sentence in the abstract and a new paragraph in the discussion addressing these points have been added.

5) I think a table stating the demographic characteristics and some laboratory data of patients and controls should have been given.

A more detailed description of the cases and controls subjects is now included in the revised version of the manuscript.

Minor Essential Revisions:
There are several errors in the language of the manuscript. Some of the mistakes are listed below:

• At page 5, the word “of” (given in red) should be added to the following sentence: For every polymorphism tested, genomic DNA of twenty individuals carrying no mutations, twenty heterozygotes and twenty homozygotes for rs1050450 were analyzed...

Done.

• At page 5 the word “text” should be corrected as “test” in the following
The intergroup comparison values and the significance of the gene-dose effect were calculated by using the chi-square test or the Fisher’s exact test.

Done.

• At page 6, the word “on” should be removed from the following sentence: To our knowledge, only two previous reports addressed on the possible role of PON1 polymorphisms in the risk for brain tumors...

Done.

• At page 6, the following sentence should be corrected for grammar: In the present study, we found not significant differences neither in the frequencies of PON1-55 and PON1-192 genotypes, nor in the frequencies of the allelic variants of these polymorphisms in patients with meningioma and grade II/III astrocytoma when compared with healthy control.

Done.

Reviewer #2:

The manuscript “Paraoxonase 1 (PON1) polymorphisms are not related with the risk for brain astrocytoma and meningioma” is a well English written document and shows data enough to demonstrate absence of correlation between PON1 polymorphisms and brain astrocytoma and meningioma. The study is well supported with the bibliographic data enclosed and also is well supported by statistical analysis.

Thank you. No changes required.

I think it would be better to increase the number of patients in order to improve the validity of the data, even though the statistical analysis shows an apparently enough powered size sample. Anyway the authors mention this aspect as a limitation of the study (discussion).

We stated in the discussion that sample size is a weakness of the study. Unfortunately this study was carried out in DNA from patients who participated in another study by our group (Olivera, 2006 #49) (now specified in the methods section), and we had limitations because we could not increase the sample size and because of DNA shortage.

The authors should improve English redaction of the abstract, particularly the second paragraph of the Background/objectives.
Thank you for the suggestion. The English redaction was revised carefully.

The statistical analysis of data is well performed, however, considering that the groups are non homogeneous in terms of gender and age, particularly patients in comparison with controls, this statistics should be performed with adjusted data (i.e. ORa = Odds Ratio adjusted by gender and age). This issue could be relevant and could change conclusions of the work.

We appreciate the suggestion. We calculated the adjusted OR for age and gender and the conclusions of the work did not change. For that reason, and because other reviewers suggested not to correct intergroup comparison values, we left the crude values in the Tables. We included a sentence in the results section indicating that even after correction for age, gender, or educational level, the intergroup comparison values remained non-significant.

Reviewer #3:

1. Are all cases Caucasian Spanish as were controls? Cases of any other race or ethnicity should be excluded from all analyses because PON1 genotype differs by race and ethnicity.

Thank you for the observation. All participants were Caucasian Spanish individuals. This is stated in the revised version.

2. Controls are younger than cases, and by design controls are largely college-educated. It is possible that genotype proportions differ by age (change over time), or even by education (this has been observed in non-Spanish Caucasians), so both age and education should be considered as potential confounders. Adjust for these in the comparisons, or state that adjustment made no difference in odds ratios. Examination of statistical significance or use of backward elimination is not sufficient to address whether confounding affected odds ratios.

As requested by reviewer #2, we calculated adjusted ORs to avoid the effect of putative confounders such as gender or age, and these confounders did not modify the results or the conclusions of the study. To assess the putative effect of education, we adjusted again the ORs by stratifying patients and control subjects into two categories: those individuals who received high education and those who did not. Again, this adjustment made no difference in odds ratios. As indicated by the reviewer, this is stated in the results section.

3. How comparable are controls to cases? Specifically: A) Were any exclusions made of “healthy” controls that may not have been made of cases, either outright (e.g. PON1-related conditions like heart disease) or built into the control selection method (healthy enough to be enrolled in college or employed as a professor)? B) Were cases from the same region(s) as
controls? This would mainly be an issue if PON1 genotype likely differed between regions of Spain (e.g. between Madrid and Badajoz), or between Spain and Portugal.

A more detailed description of the cases and controls subjects is now included in the revised version of the manuscript.

4. Remove all results, including Table 1, in which the two very distinct tumor types are combined.

Only this reviewer requested to remove Table 1. We believe that this Table is informative although distinct tumor types are combined.

5. For the genotype ORs, a non-linear model is imposed even though based on their known function, these polymorphisms would likely act in a linear fashion.

Dominant or recessive models (as is assumed for the homozygous ORs) might possibly be defensible. However, use of both homozygous wildtype and homozygous mutant individuals combined together as the reference group for the heterozygotes’ ORs is not. (Although the reference group is not shown or stated as is customary [please include], one can recreate the ORs presented and determine that the reference group for the 55 Leu/Met individuals is the Leu/Leu and Met/Met homozygotes combined together; and likewise for Q192R.)

Thank you for the observation. We calculated the ORs for the dominant and recessive models and we modified the Results section to include the results for all comparisons.

6. Include the following study of adult brain tumors and PON1 genotype in the Discussion:

Done

Minor essential revisions:

1. Abstract (Background): The second sentence is “methods” but is very similar to what is already stated there.

The text has been rewritten.

2. Abstract (Methods): More detail is required. Are these adults only, or were some children included? Where were the cases identified? Where were the controls obtained? What is the race and ethnicity of cases and controls? When were cases diagnosed?

The text has been rewritten to include the requested information.
3. Abstract (Results): Because of the small sample size, it is uninformative to simply state there were no statistically significant differences. Therefore, also indicate that the genotype frequencies were similar when comparing cases and controls.

The text has been rewritten to include the requested and additional information.

4. Abstract (Conclusions): Only two PON1 SNPs were investigated, so the authors cannot conclude that “PON1 polymorphisms” are not related with brain tumors. (The authors could indicate that “common amino acid changing polymorphisms in PON1” are not.)

The text has been rewritten.

5. Use the accepted format of the polymorphism names (Q192R and L55M).

Done. Thank you for the suggestion.

6. Introduction (paragraph 3): A) it would be more accurate to state that “some insecticides” rather than “pesticides” (much broader) are metabolized by PON1; and B) “PON1” is incorrectly italicized when referring to the PON1 protein (italics only apply to the PON1 gene).

The text has been rewritten.

7. Introduction: The description of the functional effect of Q192R is not as relevant as a discussion of the effect of this amino acid change on chlorpyrifos and diazinon, and preferably in vivo not in vitro (PON1 is not important to the in vivo disposition of parathion [paraoxon]). Also, it would be helpful to indicate whether the L55M has any known functional effect due to the amino acid change. (See Table 1 in Searles Nielsen et al. 2010, cited below.)

The text has been rewritten.

8. Methods: Cases need to be described with much greater detail. Where were they identified? What does “unselected” mean – were there any exclusions due to not agreeing to participate, too sick to provide a blood sample, already deceased (survival may be associated with genotype), etc.? When were cases diagnosed? Where did they originate from – were they all living in the region of Badajoz as were the controls? What years were they diagnosed? How was the diagnosis verified? What was the age range (to indicate whether any children or elderly individuals were included)? What was their race and ethnicity (all should be Caucasian Spanish like controls)?

With unselected we meant that all consecutive patients were included in the study group. Because this is now specified in the revised text, the word unselected was removed.

No patients were excluded. All agreed to participate.
The cases were diagnosed between the years 1997 to 1999 in the central area of Spain. The diagnosis was verified by histologic analysis. This is now stated in the revised manuscript.

9. Methods: Controls and their recruitment need to be described in greater detail so the reader can determine whether they represent a reasonable comparison group. (See all comments in major comment #3).

A more detailed description of the cases and controls subjects is now included in the revised version of the manuscript.

10. Discussion (paragraph 2): “Primitive [not primary] neuroectodermal tumors”. Note also that the cited study of childhood brain tumors has been expanded to include more children and more PON1 polymorphisms (Searles Nielsen S, McKean-Cowdin R, Farin FM, Holly EA, Preston-Martin S, Mueller BA. Childhood brain tumors, residential insecticide exposure, and pesticide metabolism genes. Environ Health Perspect. 2010 Jan;118(1):144-9). Because these analyses were in children, they need not be described in detail. Comparison of the present work should mainly focus on prior studies in adults.

The text has been rewritten and the new study has been included.

11. Discussion: The potential for bias based on the manner in which cases were identified/included, and controls were obtained, should be addressed, at least briefly. (See major comments 1-3, above.)

These potential biases have been analyzed in the revised version (see results).

12. Discussion: In the absence of exposure to chlorpyrifos and diazinon, one would expect no association between PON1 genotype and brain tumors. Therefore it would be useful to mention the extent to which cases and controls were likely exposed to these agents, either occupationally, residually or via diet. It is a limitation that the study was unable to consider this, as the above study in children observed an interaction despite there being (as in the present work) no difference in genotype overall.

Actually the mentioned study did not identify a significant interaction for the nonsynonymous PON1 polymorphisms analyzed in our study. Therefore it is unlikely that exposure would modify the findings or the conclusions in our study. Nevertheless, we included a new sentence stating this limitation.

13. Discussion (last paragraph): Removed reference to statistical power to observe an OR #1.8 for an association with both tumor types combined. Presentation of the subtype-specific power calculations is a strength and nicely included here. Those in the Methods section could be deleted.
The paragraphs in methods and in discussion sections were deleted as requested.

14. As noted earlier, it would be ideal if additional PON1 SNPs could be included; without this, an inability to include other functional PON1 SNPs and/or PON1 activity measurements should be stated as a limitation.

A sentence stating these limitations has been included in the revised manuscript.

15. A few minor typographical errors are present.

The manuscript was revised.

Discretionary Revisions:

1. Given the small sample size, the title is rather strongly worded.

The title was modified.

2. Generally the gene, not the enzyme product, is “polymorphic” (see abstract and paragraph 3 of the introduction).

The text has been rewritten.

3. The “odds ratios” for the alleles are not necessary. Further, they are essentially presented in duplicate (the OR for 55 Met is simply the inverse of the OR presented right above it for 55 Leu; and likewise for Q192R).

The Tables have been corrected to avoid duplicate data presentation. OR for minor alleles only are included.

4. Bonferroni correction seems unnecessary since so few comparisons were made in these analyses, especially since few/none were likely statistically significant even without the correction.

The sentence was modified.

Reviewer #4:

1. the control group had a men age lower than the case group (44.5± 12.2 vs 51.7 ± 17.4 and 62.1 ± 11.7 for control and astrocytoma and meningiona brain groups, respectively). The control group is not probably matched for age to the cases (a statistical analysis should be performed). This means that could be among the controls subjects that could develop a brain tumor.
It is possible that some healthy subjects would develop a brain tumor in the lapse between the mean age for controls (44 years) and the mean age for patients (52 years). Given the incidence of brain tumors, less than one out of the 220 control subjects can be expected to develop brain tumors. This would not influence the results in this study, whatever could be the PON1 genotype in this putative “false-control” subject. Moreover, following the suggestion of reviewer #2, we calculated OR corrected for age and the findings or the conclusions of the study did not change.

2. A statistical analysis should be performed to assess the possible departure from the Hardy-Weinberg law (Trikalinos TA et al., Impact of violation and deviations in Hardy-Weinberg equilibrium on postulated gene-disease associations; Am J Epidemiol 2006;163:300-9). The authors declare that “the genotypes and allele frequencies between brain tumors patients and health subjects were in Hardy-Weinberg’s equilibrium”.

Hardy-Weinberg equilibrium (HWE) was analyzed with the DeFinetti program (http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl). No departures from HWE were observed. The p values were as follows: PON1 L55M: cases, p = 0.620, controls, 0.121; PON1 Q192R: cases, p = 0.998, controls, p = 0.524. This information has been added in the revised version.

3. The authors should be report more cautiously the sample size calculation. The statistical analysis with a p value of 0.05 is much debatable since the discussion regarding the relation between p value and sample size in allelic association studies with complex disease is in progress (Zondervan KT et al. The complex interplay among factors that influence allelic association; Nat Rev 2004; 5:89-101; Colhoun HM et al. Problems of reporting genetic associations with complex outcomes, Lancet 2003;361:865-72). I believe that in this study a possible negative false results it can be not excluded.

We recalculated all p values by the use of FDR and permutation tests. As expected, none of the comparisons became statistically significant. We agree with the reviewer that in this study we cannot exclude a false negative result due to the sample size. This is acknowledged in the discussion section.
We hope that you consider the revised version of the manuscript adequate for publication in BMC Neurol.
We are looking forward to hear from you.

Yours sincerely,

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