Author's response to reviews

Title: NQO1 gene rs1800566 variant is not associated with risk for multiple sclerosis

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Version: 2  Date: 31 March 2014

Author's response to reviews: see over
Reviewer's report
Title: NQO1 gene rs1800566 variant is not associated with the risk for multiple sclerosis
Version: 1 Date: 26 February 2014
Reviewer: Giulio Disanto

Reviewer's report:
Agundez et al. aimed to investigate the potential association between a genetic polymorphism located in the NQO1 gene (involved in oxidative stress) with risk of multiple sclerosis (MS), age of onset, gender and clinical type of MS. This gene is not included in the list of MS associations provided by large GWAS in MS. However, a previous Greek study has reported the presence of an association, therefore it seems reasonable to attempt to replicate their findings. In contrast with the Greek study, Agundez et al found no evidence of association. I believe it is important to report negative studies to provide evidence against the large number of published false positive associations. However, I have a number of concerns that should be addressed.

MAJOR COMPULSORY REVISIONS
1) The quality of English across the manuscript is not great and needs to be improved.
OK. The manuscript was revised by a native expert.

2) In the introduction, the authors say that the majority of MS genetic associations are associated with other autoimmune diseases. However, based on the latest GWAS in MS (Beecham et al. Nature Genetics), only “~22% of signals overlapped at least one other autoimmune disease signal”. Please correct
OK. Corrected.

3) Table 1: could you please clarify whether each finding comes from EAE or MS? For some studies, this was not clear. Having an additional column providing this information would be helpful.
OK. Done. This table has been included in a supplementary file and removed from the main manuscript.

4) Were all individuals included in the study of Spanish Caucasian origin? How was ethnicity assessed?
Ethnicity was based on self-report. This information has been included in the revised manuscript.

5) How was the association between the SNP and risk of MS assessed? Was it done using logistic regression? The author mention Chi Square, Fisher, t test, Mann-Whitney but none of these statistical tests provide OR with 95% CI. I think the authors used logistic regression but this information should be included.
OK. The associations were based two-way contingency table analysis, which provide OR and 95% C.I. This information has been included in the revised manuscript.
6) The authors provide a power calculation assuming an OR=1.5. Can the authors please provide a power calculation for OR=1.1, 1.2, 1.3 and 1.4? This is important to understand how sensitive this analysis was to detect a difference. Also, are the sample size and statistical power in this study greater than in the Greek study? If so, this validates your results and should be commented. AND 7) The authors say that the power of the study was 84.6% and 90.9% for bilateral and unilateral associations respectively. Can the authors clarify what they mean by unilateral and bilateral?

The power calculations requested are the following:

<table>
<thead>
<tr>
<th>O.R.</th>
<th>One-tailed (present study)</th>
<th>Two-Tailed (present study)</th>
<th>One-tailed (Greek study)</th>
<th>Two-Tailed (Greek study)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>90.92%</td>
<td>84.61%</td>
<td>90.38%</td>
<td>83.94%</td>
</tr>
<tr>
<td>1.4</td>
<td>79.03%</td>
<td>68.86%</td>
<td>78.41%</td>
<td>68.23%</td>
</tr>
<tr>
<td>1.3</td>
<td>59.84%</td>
<td>47.37%</td>
<td>59.39%</td>
<td>47.02%</td>
</tr>
<tr>
<td>1.2</td>
<td>36.61%</td>
<td>25.55%</td>
<td>36.44%</td>
<td>25.47%</td>
</tr>
<tr>
<td>1.1</td>
<td>16.56%</td>
<td>9.91%</td>
<td>16.56%</td>
<td>9.93%</td>
</tr>
</tbody>
</table>

The patient’s sample size in our study is greater than in the Greek study. The statistical power in our study is slightly greater than that in the Greek study.

The text has been corrected to include this information and the terms unilateral and bilateral have been changed to one-tailed and two-tailed, respectively.

8) Table 2: EDSS is an ordinal variable and it does not make much sense to provide mean and SD. Provide instead median and IQR. OK. Done.

9) Table 3: It is not clear to me how the authors calculated the OR and which genotype and allele were used as reference categories. In the legend, the authors write that “Major alleles and genotypes were assumed as reference values”. This is ok, but then I do not understand the meaning of the OR and 95%CI of CC genotypes and allele C. These are supposed to be the reference categories. What were they tested against? In any case, there is no reason to perform the same test twice (e.g. if you test the effect of allele T using allele C as reference, there is no need to test the effect of allele C using allele T as reference. These two tests are equal and indeed the p value is the same). The authors should simply use logistic regression with major alleles as reference category and provide the OR, 95%CI and p values obtained for the minor allele. Ok. Done

10) In the text the authors say that “allelic and genotype frequencies were not influenced by gender”. If I look at table 3, it seems the authors compared genotypic and allelic frequencies between male cases vs male controls and female cases vs female controls. If so, this is not testing whether frequencies are influenced by gender. This means testing whether the association between this SNP and risk of MS is influenced by gender which is not exactly the same. Please correct the sentence in the text. OK. The text has been corrected.
11) Please provide details about each test performed (e.g. age, EDSS and progression index stratified by genotype with the corresponding p values).
OK. Done.

12) The authors say that this SNP is classified as “pathogenic”. I believe the authors need to clarify to what extent the allele T is pathogenic? Based on what evidence? Which disease? It has anyway a high frequency in the general population.
The evidence is available by following the link at the end of the sentence. The classification is based on the clinical impact of each SNP. The supported values are:

- unknown
- untested
- non-pathogenic
- probable-non-pathogenic
- probable-pathogenic
- pathogenic
- drug-response
- histocompatibility
- other

This SNP has been linked to altered susceptibility to benzene toxicity and response to chemotherapy (see http://browser.1000genomes.org/Homo_sapiens/Variation/Phenotype?db=core;r=16:69744645-69745645;v=rs1800566;vdb=variation;vf=1366399 for further details). This comment has been added in the discussion.

13) The following sentence is not clear: “The present study has some limitations. First, the size of analyzed cohorts may not be sufficient for strict conclusions about NQO1 role in MS. Second, despite the sample size is adequate to detect an OR as small as 1.5, a more modest association would not be detected (this is a usual weakness of genetic association studies). Third, because the cohort study included MS patients with different degrees of severity, it is not adequate for the investigation of the influence of NQO1 genotypes on the disability or severity of MS (the ideal study for this purpose should include genotyping of patients with a recent diagnosis of MS with similar follow-up periods)”. The first two limitations are actually the same thing (i.e. the small sample size does not allow conclusive evidence because there is no power to detect smaller effects).
The third limitation is also unclear. The problem is not that there are patients with different degrees of severity (you actually need these people because if every patient had the same severity there would be nothing to test!). The problem is rather that these patients have different disease durations. Also, the authors say that the ideal cohort to test this should include patients with a recent diagnosis of MS. This is also unclear because if the diagnosis was recent, then you would not be able to distinguish between cases with slow or fast progression. Please clarify.
OK. This paragraph has been corrected to clarify these points.
MINOR ESSENTIAL REVISIONS

1) I believe the introduction is too long and the authors provide information that in my opinion is not needed. For example, there is a long paragraph describing the role of putative environmental associations in MS. This could be either deleted or reduced to a single sentence. Please give only the information that the reader actually needs to understand the paper.
   OK. Reduced

2) As for the introduction, the discussion can be considerably shortened. For example there is no need to list the IDs of all non-synonymous SNPs located within NQO1.
   OK. Deleted.

3) I suggest table 1 be included as supplementary material
   Ok. Done

4) Table 3: Are percentages with their 95%CI within the brackets? Please clarify this because it is not clear.
   OK. The tables have been changed.
Reviewer's report
Title:NQO1 gene rs1800566 variant is not associated with the risk for multiple sclerosis
Version:1 Date:28 February 2014
Reviewer:Carles Vilarino-Guell
Reviewer's report:
Agundez et al present the association of one SNP located in NQO1 with MS susceptibility risk, disease course and severity in a Spanish population. This is a rather simple study attempting to replicate some positive association previously described in MS patients from Greece. The study is well design and the conclusions drawn are overall appropriate however the length of the manuscript is excessive for the message it conveys.

1) In the abstract background the authors should mention the previously described association for NQO1 in MS, as it is one of the main reasons to do their study.
   OK. It has been mentioned in the abstract background and in the introduction.

2) Some references seem to be missing. Most noticeably: “A number or reports have suggested a possible role of oxidative stress and lipid peroxidation in the inflammatory processes and in the pathogenesis of MS.” And “The enzymatic activity of NQO1 depends fundamentally on a single nucleotide polymorphism (SNP) at the NQO1 locus, rs1800566 (C609T), which produces a proline-to-serine substitution at amino acid 187 (P187S)”
   OK. References added.

3) At the end of the introduction the authors claim “In an attempt to identify additional factors involved in MS susceptibility, we genotyped the SNP rs1800566 in the NQO1 gene in Spanish MS patients and healthy subjects” which is inaccurate. The authors are not attempting to identify additional factors but attempting to replicate a previously described association in an independent population.
   OK. Changed.

4) Comprehensive table legends must be added to define what the data presented is. For example, in table 3 the first genotype count is 178 (61.4, 55.8-67.0), what those three numbers are escapes me. In addition these tables should be simplified to facilitate reading (suggestion: remove gender specific analysis since nothing is significant), and should provide n and % for genotype and alleles. It is also unclear what statistical method was used for each group of analysis.
   OK. This has been stated in the revised version of the manuscript.

5) The authors claim “genotype frequencies were not influenced by gender”, but they actually mean that association between the variant and disease risk was not observed when analyzing gender separately. This should be addressed.
   OK. The text has been changed.
6) They also failed to identify an association with “each MS phenotype” when they mean disease course. Although they are following the model of Stavropoulou et al, analysis of RR and SP patients separately is not appropriate (although common) as disease duration has an effect on the phenoconversion from RR to SP. We suggest the authors analyze these two groups together and call them RR onset.
OK. Corrected in the tables of results.

7) In the discussion the authors present the limitations of their study, two of those: small sample size and lower than expected OR are dependent on each other and therefore the same. We suggest the authors combine these two caveats into one. (ie. if the OR was lower than expected our sample size would not be sufficient to …).
OK. Combined.

8) Maybe I did not get enough coffee today, but I could not find table e-2. Table e-2 was not a table of this work, but of the DeFinetti software. In addition, at this time the link http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl, Table e-2 does not work. It has been changed to http://ihg.gsf.de/cgi-bin/hw/hwa1.pl. This has been changed in the text

9) Although grammatically correct, some language polishing may be required. As example: “Some experimental data suggests” would be better as “It has been suggested/proposed/…” “These data suggest that NQO1 rs1800566 polymorphism is not related with the risk for MS” would be better as “Our results indicates that NQO1 rs1800566 does not have an effect on MS disease risk” In addition, the authors used the word “related” when meaning “associated”, which should also be corrected.
Ok. Corrected. Moreover, the manuscript was revised by a native expert.

10) There are several sections of this manuscript which are not entirely relevant to the study and could be easily removed. Including from “It has been suggested that findings from…” in the first paragraph until the end of the second paragraph.
OK. Removed (this was suggested by reviewer 1 as well)

11) The last three lines of paragraph three as well as Table 1. In addition, the first paragraph of the discussion, which is a repeat of the introduction; and the third paragraph of the discussion are also irrelevant to this study.
OK. Table 1 has been included as a supplementary file and removed of the main text (as was suggested by reviewer 1). The list of the IDs of all non-synonymous SNPs located within NQO1 has also been deleted as was suggested by reviewer 1

12) Similarly, sentences such as “This enzyme is encoded by the NQO1 gene (chromosome 16q22.1, Gene Identity 1728) (link http://www.ncbi.nlm.nih.gov/gene/1728)” Appear unnecessary and should be excluded.
We believe that this information may be useful to readers and, provided that the electronic journals have no space limitations, we decided to keep it.

13) Or “Genotyping for rs1800566 allelic variant was performed … using TaqMan Assays (C___2091255_30, Life Technologies, Alcobendas, Madrid, Spain) designed to detect the SNP rs1800566”; in this sentence “allelic variant” and “designed to detect the SNP rs1800566” is redundant. OK. Deleted.

14) Also “Mean age at onset of MS did not differ significantly between patients carrying NQO1 rs1800566 C/C (mean + SD = 32.1 ± 10.2 years), C/T (mean + SD = 33.5 ± 11.6 years) and T/T (mean + SD = 32.4 ± 12.0 years), (p= n.s. for the comparison of carriers vs non-carriers of variant alleles). The last section in brackets is redundant. OK. This paragraph has been changed according with the comments of reviewer 1 (“provide details about each test performed e.g. age, EDSS and progression index stratified by genotype with the corresponding p values”)

15) The authors should be able to substantially improve this manuscript and make it more concise and easy to read by carefully addressing these little issues, and removing unnecessary paragraphs from the introduction and discussion. OK. Done in agreement with previous comments.

16) We also suggest the authors do not refer to the study by Stavropoulou et al as “the Greek study”. OK The text has been changed
Reviewer's report
Title: NQO1 gene rs1800566 variant is not associated with the risk for multiple sclerosis
Version: 1 Date: 6 March 2014
Reviewer: Julia Pakpoor
Reviewer's report:

Major revisions:
1) The limitations need further discussion. The authors state that the SNP investigated in this study shows a relatively high minor allele frequency (20% in caucasians). The authors go on to state that the study is adequately powered to detect an OR as small as 1.5. The vast majority of MS associated SNPs have ORs much smaller than this. In light of how common the minor allele frequency of this SNP is and the fact that this is not already an established MS associated SNP, would they not expect, if there is a true positive association, an OR much smaller than 1.5 and therefore perhaps not detectable by the sample size used in this study. Actually this is addressed in the sentence “although adequate to detect an OR as small as 1.5, a more modest association would not be detected”

Minor essential revisions:
1) The manuscript requires grammatical proof-reading throughout. OK. The manuscript was revised by a native expert.
2) The background part of the abstract makes no justification for the choice to study SNP rs1800566. It is important to include this so that readers can gain an understanding of the project from the abstract. OK. Changed as it was also suggested by reviewer 2.
3) “affecting the Central Nervous system” - no need for capital letters for central nervous system. – OK, corrected. please correct “confounder factor” to “confounding factor” After shortening the document as it was suggested by reviewers 1 and 2, this term was not used in the manuscript.
4) - please write out the abbreviations “OR” and “EDSS” on first use OK. Done.

Discretionary revisions:
1) “vitamin D status” is unclear, vitamin D levels may be more accurate. After shortening the document as it was suggested by reviewers 1 and 2, this term was not used in the manuscript.