Author's response to reviews

Title: The intron 4c allele of the NOS3 gene is associated with ischemic stroke in African Americans

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Author's response to reviews: see over
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Dear Dr. Kowalczuk:

We are grateful to the reviewer for the comments regarding our manuscript entitled “The intron 4c allele of the NOS3 gene is associated with ischemic stroke in African Americans”.

We are gratified that he feels that “Overall, this study represents an important contribution to the literature validating previous observations”.

We have revised the manuscript taking into account the criticisms.

Major compulsory revisions:

1-Methods-The authors should provide a more detailed description of their statistical methods, including which covariates were considered and how the final covariates were selected for inclusion in their adjusted model. In particular, please clarify why cigarette smoking status and gender were not included, particularly since cigarette smoking status has been shown to modify the relationship between genetic polymorphisms in NOS3 and various cardiovascular phenotype (endothelial function, ischemic stroke risk, CHD risk, etc.).

We revised the manuscript and provided a more detailed description of our statistical methods. We included gender in the regression model in the revised version of manuscript and it did not change the results. Genetic polymorphisms in NOS3 remained to be significantly associated with stroke in the pooled analysis of all patients and in American Africans (p<0.05). Smoking status was not included since it was not significantly related to disease and was excluded from the logistic regression analysis. We added a sentence in the last paragraph of method section, which read “ Covariates in the regression model included age, gender, diabetes and hypertension. Smoking status (ex- and current smoker vs. non-smoker) was not significantly related to disease and was excluded from the logistic regression analysis”. We also revised the Table 3 accordingly.
2-The authors need to clarify why genotyping data for the Glu298Asp polymorphism was only obtained in a fraction of the population (missing in 32% of controls and 13% of cases), particularly if there were no genotyping failures. This needs to be addressed as a limitation in their analysis, which could have contributed to the lack of an association observed with ischemic stroke risk.

The initial intent of this study was to focus on analysis of the intron 4 allele in order to replicate previous results in the Chinese population (ref 4 of the manuscript). The data on any association between Glu298Asp and stroke has been conflicting; a positive result was found in French patients but only with lacunar stroke. No association between ischemic stroke and the polymorphism was found in Scottish or British patients. The primary purpose of including this polymorphism in our analysis was to determine if any of the intron 4 allele/Glu289Asp haplotypes were associated with stroke. As with most laboratories, resources/time are limited and based upon the preliminary analysis performed during the course of the study, the decision was made not to continue any further genotyping of the Glu289Asp polymorphism. However, a sentence has been added at the end of the first paragraph of the Discussion section indicating the possible limitations of our negative result due to a small sample size.

3-The authors should add a paragraph to the Discussion commenting on the limitations in their analysis. This should include issues related to the small sample size and limitations in statistical power, particularly with the rare intron 4c allele.

This has been done, last paragraph in the Discussion section.

Minor Essential Revisions:

1-The authors should use the term “race” not “ethnicity” throughout the manuscript to reference analyses across White/Caucasian and African-American individuals. Also, please clarify how race was defined (ie, was this by patient self-report?).

Throughout the manuscript, race is now used. Also, a clarification in the Methods section is provided regarding the definition of race.

2-Results, paragraph 5-The authors should state that the intron 4c allele is rare in both African-Americans and Caucasians, and the results should be interpreted with caution in both racial groups (the allele counts in the control group are only 2 for each racial group).

We agree that it is important for readers to be aware of the small sample size with respect to intron 4c; however, we already added a paragraph to the Discussion (Major Compulsory Revision #3 above) section emphasizing the limitations of this portion of the study.
3-Discussion, final paragraph-The authors attempt to pool the data from their analysis and reference #14 to obtain a “pooled” odds ratio. There are numerous limitations to such an approach, and this should be removed from the Discussion. The authors can simply state the OR from each individual study, and comment on the consistency of the observed relationships across population.

We have removed the combined analysis (paragraph #4 of the Discussion section) as suggested.

4-Table 2-Was the difference in the Glu298Asp T allele frequency in African-American cases (26.2%) and controls (15.4%) not statistically different? It may be helpful to add the regression analysis for the Glu298Asp polymorphism to Table 3, to more comprehensively present the data for the reader.

The difference in the Glu298Asp T allele frequency was not statistically different between African-American cases and controls. We agree with the reviewer and added the regression analysis of the Glu298Asp polymorphism to Table 3.

Discretionary Revisions:

- Introduction and Discussion- When discussing prior studies evaluating the relationship between genetic polymorphisms in NOS3 and ischemic stroke, the authors should clarify whether presence of the described variant alleles was associated with higher or lower risk of stroke. Moreover, the authors should include the well-characterized- 786T>C (rs2070744) polymorphism in this discussion, particularly since it is functionally relevant and has been associated with risk of ischemic stroke in other populations. The authors should also justify why this polymorphism was not evaluated in their analysis.

The results of prior studies are described in the Discussion section of the paper. We feel that it would be redundant to discuss these results twice. Although the 786 T>C allele is an interesting polymorphism in the NOS3 gene, it was not studied in this project. We plan to study it in our population in the future.

- Discussion, final paragraph- The authors reference #14 as the only report evaluating the NOS3 gene in a biracial population; however, a recent publication evaluating the association between the Glu298Asp and 786T>C polymorphisms and risk of incident ischemic stroke in the ARIC population has been published (Lee et al. Pharmacogenet Genomics. 2006;16:891-99). This analysis should be cited and considered in data interpretation.
We appreciate the reviewer making us aware of this important contribution to the NOS3 literature, however, given that the intron 4 allele was not studied in that report, we are not sure how to consider it in our data interpretation.

- Table 1- Please include the racial distribution, independent of genotype, across the case/control groups. Please also include information on cigarette smoking status. Lastly, please provide a footnote describing what the allele counts refer to. I presume ‘2n’ are allele counts, but this needs to be clear for the reader.

We agree with the review’s opinion and listed the racial distribution and smoking status in Table 1 of our revised manuscript. We also added a footnote to Table 2 to describe the definition of “2n” in the table.

As requested in your email letter, we have provided a power calculation regarding the strength of the association which has been indicated in the results section of the abstract.

We thank the reviewer for his comments, many of which have been incorporated into this revised manuscript. We feel that the manuscript is improved.

We hope the changes are acceptable.

Sincerely:

Raji Grewal MD,
Associate Professor of Neurology