Author's response to reviews

Title: Association of the eNOS E298D polymorphism and the risk of myocardial infarction in the Greek population

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Author's response to reviews: see over
Rebecca Simmons
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Re: MS 1265843313210430

Dear Mrs Simmons,

Thank you for reviewing our manuscript. We have revised the paper according to the recommendations of the reviewers. Detailed responses to each comment are enclosed. I would like to re-submit our manuscript entitled “Association of the eNOS E298D polymorphism and the risk of myocardial infarction in the Greek population” hoping that it is now acceptable for publication in the journal “BMC Genetics”.

This article presents original work, not previously published and is not under consideration elsewhere in any language.

I thank you in advance.

Yours sincerely,

Nikolaos Drakoulis, MD, Ph.D.
Corresponding Author
RESPONSE TO COMMENTS

REVIEWER #1 (Anke Tonjes):

**Major issues**

**Comment 1:**
The reproducibility and validation of the genotyping method should be provided by sequencing of the PCR product or genotyping of duplicates.

*Answer:*
The issue of the reproducibility and validation of the genotyping method is fully covered under the ISO 17025 certificate of the laboratory where the analysis took place. A validated LightMix (TIB-MOLBIOL) was used for the detection of the eNOS E298D SNP. The kit contains control DNA (human eNOS 298D mt, wt, hetero $10^5$ target equivalents per reaction). In addition, to confirm reproducibility, mutation analysis of 9 random samples (3 wt/wt, 3 wt/mt and 3mt/mt) was performed 10 times each. The results were confirmed for homozygous mutant, homozygous wild type and heterozygous samples in all repeated measurements.

**Comment 2:**
The association of the eNOS E298D variant with MI risk should be assessed by logistic regression with appropriate adjustment for all other risk factors such as smoking, hypertension, hypercholesterinaemia etc.

*Answer:*
Logistic regression analysis with appropriate adjustment for possible risk factors has been performed as requested (see table 4 - updated version). Following your suggestion, we decided to use multivariate analysis, thus we excluded from the article tables 4 and 5 (previous version).

**Comment 3:**
Genotypic effects should be assessed in a classical additive, recessive and dominant mode of inheritance

*Answer:*
Additive genetic effects occur when the combined effects of alleles at different loci are equal to the sum of their individual effects. This was meant on page 7 of the manuscript: The production of
NO is disturbed by the eNOS E298D SNP (i.e. one Thymine instead of one Guanine at position 894 in exon 7 of their eNOS gene). As a result, individuals having this mutation would no longer have full vasoprotective effects of NO. Platelet aggregation, leukocyte adhesion and smooth muscle cell proliferation would not be suppressed enough and would be added to other risk factors, such as hypercholesterolemia and others, leading finally to higher incidence of MI. Whether the T or the G allele is dominant or recessive is not clearly documented yet but it is known that individuals having the TT genotype produce less NO than either the GT or the GG individuals (JP Casas et al. Endothelial Nitric Oxide Synthase Gene Polymorphisms and Cardiovascular Disease: A HUGE Review. Am J Epidemiol 2006; 164: 921–935). Consequently, in order to avoid confusion we preferred to omit the aforementioned terms and the corresponding sentences.

Comment 4:
The authors should provide a power calculation and compare the power of the presented study with published data.
Answer:
A relevant statement comparing the power of the current study with existing literature requirements has now been added in the text (first paragraph in Statistical Analysis section).

Comment 5:
The manuscript would benefit from a meta-analysis of the effect in the current study and published data
Answer:
A recent meta-analysis (Am J Epidemiol 2006; 164:921–935) including a variety of pertinent studies in different ethnicities indicates a wide variability in the occurrence of the 298D mutation with regard to cardiovascular diseases. Almost the same variability is noticed when we focused on the MI studies of Caucasian population only.
For more details please see Web appendix tables 1, 2, and 3 of JP Casas et al. Endothelial Nitric Oxide Synthase Gene Polymorphisms and Cardiovascular Disease: A HuGE Review in Am J Epidemiol 2006;164:921–935 describe genotype frequencies in apparently healthy subjects from 64 sample populations, divided according to ethnic background. (This information is presented in the first three of six supplementary tables; each is referred to as “Web appendix table” in the text.
and is posted on the website of the Human Genome Epidemiology Network (http://www.cdc.gov/genomics/hugenet/reviews.htm) as well as on the Journal’s website (http://aje.oupjournals.org/).

As the above information creates no special demographic differentiation with regard to the variability of our population (all Greeks patients involved in the study were Caucasians) we did not consider it necessary to include such data in the paper.

**Minor issues**

**Comment 6:**
Test-statistics are difficult to interpret for the reader and should be left out. For all tests effect sizes and directions should be provided along with the p-values.

*Answer:*
We have reviewed the necessity of the presentation for all statistic tests and some of them were left out. We also made the required arrangements / additions for p-values where applicable.

**Comment 7:**
Please provide a legend for each table.

*Answer:*
Each table is now accompanied by the relevant, appropriate legend.

**Comment 8:**
The presented figures are not essential and should be omitted.

*Answer:*
We agree that Figures 1 and 2 are not essential and thus they have been omitted.

**Comment 9:**
Please standardize positions after decimal point in the tables

*Answer:*
The position for all decimal points in the tables as well as in the text has been standardized.
REVIEWER #2 (Stavroula Kanoni):

**Major Compulsory Revisions**

**Comment 1:**
The manuscript could benefit from language editing.

**Answer:**
We fully agree with this comment. Therefore the whole manuscript has undergone a thorough language review.

**Comment 2:**
Results. The authors should mention the power analysis, especially for the results regarding smoking, genotype, MI and gender

**Answer:**
A relevant statement comparing the power of the current study with existing literature requirements has now been added in the text (first paragraph in Statistical Analysis section).

**Comment 3:**
Discussion. The authors should mention the study limitations.

**Answer:**
A new paragraph mentioning the study limitations has been added at the end of the discussion section.

**Comment 4:**
Page 2, Abstract, Results, lines 4-7: “This risk was higher when the genetic factor was combined with other risk factors for MI,..., and positive family history.” With the exception of smoking, there are no results in the manuscript that support this phrase.

**Answer:**
We fully agree with your comment and we have omitted this phrase from the abstract accordingly.
Comment 5:
Page 4, Methods, Study population. The authors should clarify whether patients and control subjects were matched for some characteristics (e.g. age, gender, BMI).

Answer:
Matching for basic patients characteristics, such as age, gender, BMI was not initially accomplished, but controlling for those characteristics in the statistical analysis eliminates their possible confounding effect.

Comment 6:
Page 7, Methods, Statistical analysis, lines 11-14.
The authors mention that they have applied two different genetic models: additive and dominant. However, the recessive model is also presented in the results and tables. The authors should also include this in the “methods”. The authors should describe the additive model more clearly in Methods, Results and Tables 3 and 5. They present the additive model as “T allele vs. G allele”, which is a more suitable description for the allelic model. If the authors have used an allelic genetic model in their analysis instead of an additive, they should clarify that. The authors should also explain the rationale for the multiple genetic models testing (additive, dominant, recessive), included in their analysis. Was there a “best-fit” statistical approach used for the selection of the genetic model?

Answer:
Additive genetic effects occur when the combined effects of alleles at different loci are equal to the sum of their individual effects. This was meant on page 7 of the manuscript: The production of NO is disturbed by the eNOS E298D SNP (i.e. one Thymine instead of one Guanine at position 894 in exon 7 of their eNOS gene). As a result, individuals having this mutation would no longer have full vasorotective effects of NO. Platelet aggregation, leukocyte adhesion and smooth muscle cell proliferation would not be suppressed enough and would be added to other risk factors, such as hypercholesterolemia and others, leading finally to higher incidence of MI. Whether the T or the G allele is dominant or recessive is not clearly documented yet but it is known that individuals having the TT genotype produce less NO than either the GT or the GG individuals (JP.Casas et al. Endothelial Nitric Oxide Synthase Gene Polymorphisms and Cardiovascular Disease: A HuGE).
Review. Am J Epidemiol 2006; 164:921–935). Consequently, in order to avoid confusion we preferred to omit the aforementioned terms and the corresponding sentences.

Comment 7:
Page 8, Results, Distribution of the E298D polymorphism and Page 19, Table 2.
The authors must provide a p-value for the frequencies comparison. It would be helpful if the frequencies and the p-value for the recessive genetic model were also added, since the main findings of the study are based on that model.

Answer:
P-values are now provided in table 2. Therefore comparison of the two groups and inference about statistical significance can now be performed based on p-values estimates.

Comment 8:
Page 9, Results, Smoking, E298D polymorphism and MI, line 6: “…, suggesting a significant interaction between smoking and eNOS polymorphism for MI”.
The authors should mention if there was a significant p-value for the genotype x smoking interaction term, before stratifying for the smoking categories.

Answer:
Following your suggestion, we decided to examine simultaneously the study parameters, thus we excluded from the article tables 4 and 5 (previous version). When the study parameters entered to the multiple logistic regression model, we found no evidence for a possible interaction between smoking and eNOS polymorphism. The phrase you mentioned has been deleted from the text.

Comment 9:
Page 9, Results, first paragraph and Page 19, Table 3. The authors should include in the analysis adjustments for other potential confounding factors (e.g. age, gender, BMI, other cardiovascular risk factors).

Answer:
Logistic regression analysis with appropriate adjustment for possible risk factors has been performed as requested (see table 4 - updated version).
Comment 10:
Page 10, Results, Distribution of genotypes by age groups.
These results are interesting, however the authors should also present the sample size and characteristics of the two age-groups. Was the frequency of the investigated polymorphism similar in the two age-groups? Also, adjustments for other potential confounding factors (e.g. gender, BMI, cardiovascular risk factors) should be included in the presented analysis.

Answer:
Following your suggestion, we preferred to perform logistic regression analysis. Several alternative statistical models were performed and the respective findings are presented in Table 4. Therefore we decided to exclude the previous subgroup analysis by age groups.

Comment 11:
Page 11, Discussion, lines 5-8.
There is no data that support this sentence in Table 1 or anywhere else in the results. The authors should provide more data that support this.

Answer:
According to the new findings this phrase has been omitted.

Comment 12:
Page 11, Discussion, lines 2-5.
The authors should explain more clearly if their results support the recessive or the additive genetic model.

Answer:
Please see our answer in Comment 6.

Comment 13:
Page 12, Discussion, line 9: “genetic factors are more likely to affect young rather than old people…”.
The authors should provide published evidence, in order to support this sentence.

Answer:
Two previous published studies have been added as a documentation of this sentence at the relevant point of the discussion section (see references 24 & 25 in the text).

**Minor Essential Revisions**

**Comment 1:**
Page 5, Method, Control subjects, last line: “Body mass index was measured in all.”
The authors should rephrase that sentence, as body mass index is calculated rather than measured. A brief description on body weight and height assessment should be given. Were they measured or self-reported?

*Answer:*
We agree that the term “calculated” is preferable to the term “measured”, especially when body weight and height are self-reported, which was also the case in our study. Consequently, the text has been revised accordingly.

**Comment 2:**
Page 6, Methods, Coronary risk factors, first line: “Diabetes mellitus was defined by elevated blood glucose levels after fasting (>140 mg/dl)...”. The authors should clarify why they applied this cut-off point, while according to the latest guidelines diabetes mellitus is defined as fasting blood glucose levels ≥126 mg/dl.

*Answer:*
Thank you for the comment. We re-checked the hospital protocol and we confirm that all study patients were screened for diabetes mellitus based on fasting blood glucose levels of 126 mg/dl. We apologize for the typo which now has been corrected in the text.

**Comment 3:**
Page 7, Methods, Statistical analysis, fifth line: “Comparisons between subjects were made...”.
The sentence is unclear. Do the authors mean comparisons between subject’s groups?

*Answer:*
Indeed, it was meant to be “comparisons between subject’s groups” i.e cases and controls and now this has also been clarified in the text of the manuscript (statistical analysis section).
Comment 4:

Page 8, Results, Comparison of the two study groups, sixth line: “the two groups were comparable...”. The authors should use a more appropriate statistical term like “similar” or “not significant different”.

Answer:

We thank for the comment. The text at this point has been now been fully revised from a statistical point of view.