Author’s response to reviews

Title: Association of an INSIG2 obesity allele with cardiovascular phenotypes is gender and age dependent

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Author’s response to reviews: see over
We thank the reviewers for their very thorough, detailed, and fair comments, criticisms, and suggestions. We have attempted to address every concern and have revised the manuscript accordingly. We believe the manuscript has been greatly improved and look forward to review of the revision. Thank you.

Reviewer 1:

**Comment:** The manuscript is difficult to read and the reasoning for a number of steps in the manuscript is not clear. The author uses overly long and elaborate sentence structure. The article could be shortened into a more succinct form.

**Response:** We have simplified sentence structure and tried to make more clear the reasoning for the analysis.

**Comment:** It is not clear if they looked at one or several SNPs. If so then a haplotype and linkage analysis would be required.

**Response:** Only one SNP was analyzed.

**Comment:** It is not clear what the primary aim of the original analysis was. It is presumed that the authors had chosen CAD as the original primary phenotype. The report of the secondary phenotypes of PAD and CV in different age and sex cohorts suggests that these results were obtained after many different analysis using different vascular phenotypes as the dependent variable. This can be appropriate but the issue of multiple comparisons as well as selection bias becomes important and must be addressed in the methods section.

**Response:** Because the population all had CAD, our primary phenotypes were those presented in Table 3. We analyzed all simultaneously and have modified Tables 3 and 4 to include Bonferroni adjusted p-values to correct for multiple comparisons.

**Comment:** Additionally was this the only SNP for which associations where tested.

**Response:** Yes, only one SNP was analyzed.

**Comment:** The authors did a simple Chi square and did not do any logistic regression or multi-factorial analysis. It would have been important to control for sex, age, HTN, diabetes and in this case BMI to control for confounding errors in the analysis. Controlling for these issues may have pushed the results into the significant range.

**Response:** A stepwise logistic regression model has been added to estimate the independent effects of different variables. The strengths of association were indeed increased after this analysis.

**Comment:** The authors did not describe whether the model they used was base on a dominant, codominant or recessive model. It appears the final assessment assumed codominance.
Response: We used a dominant model in which the presence of one or both risk alleles was considered as a separate group from those without a risk allele.

Comment: Did the authors address the issue of admixture and did they segregate their genetic analysis by race? It is not clear in the manuscript that this was done. If not then why not, and this should have a significant effect on the analysis. If this analysis was not done then it must before the article can be accepted.

Response: We did not segregate the population by race since the number of non-Caucasian patients did not provide sufficient power. We do note that very little difference in allele frequency was present in HapMap (www.hapmap.org) data among Caucasian and African populations. The tables below are now included in the manuscript.

<table>
<thead>
<tr>
<th>Total</th>
<th>CC</th>
<th>GC</th>
<th>GG</th>
</tr>
</thead>
<tbody>
<tr>
<td>(N=947)</td>
<td>(N=94)</td>
<td>(N=413)</td>
<td>(N=440)</td>
</tr>
<tr>
<td>White</td>
<td>85 (10.9%)</td>
<td>345 (44.3%)</td>
<td>348 (44.7%)</td>
</tr>
<tr>
<td>Black</td>
<td>8 (5.9%)</td>
<td>50 (36.8%)</td>
<td>78 (57.4%)</td>
</tr>
<tr>
<td>Asian</td>
<td>0 (0%)</td>
<td>5 (50%)</td>
<td>5 (50%)</td>
</tr>
<tr>
<td>Other</td>
<td>1 (4.3%)</td>
<td>13 (56.5%)</td>
<td>9 (39.2%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Population</th>
<th>allele</th>
<th>freq</th>
<th>count</th>
<th>allele</th>
<th>freq</th>
<th>count</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caucasian</td>
<td>C</td>
<td>0.331</td>
<td>515</td>
<td>G</td>
<td>0.669</td>
<td>1041</td>
<td>1556</td>
</tr>
<tr>
<td>AA</td>
<td>C</td>
<td>0.243</td>
<td>66</td>
<td>G</td>
<td>0.757</td>
<td>206</td>
<td>272</td>
</tr>
<tr>
<td>Utah</td>
<td>C</td>
<td>0.265</td>
<td>60</td>
<td>G</td>
<td>0.735</td>
<td>166</td>
<td>226</td>
</tr>
<tr>
<td>Kenya</td>
<td>C</td>
<td>0.275</td>
<td>78</td>
<td>G</td>
<td>0.725</td>
<td>206</td>
<td>284</td>
</tr>
<tr>
<td>Nigeria</td>
<td>C</td>
<td>0.230</td>
<td>52</td>
<td>G</td>
<td>0.770</td>
<td>174</td>
<td>226</td>
</tr>
</tbody>
</table>

Comment: Finally the authors state the population is in hardy Weinberg equilibrium. They state that this was done with a helix tree software package but not the markers used nor the technique. If their analysis included all racial subtypes it is not likely that they were in hardy Weinberg equilibrium. Did they use a sophisticated marker analysis?

Response: The INSIG2 SNP was analyzed in the entire cohort and found to be in Hardy Weinberg equilibrium. These results are consistent with the similar allele frequencies from the major racial sub-group as discussed above.

Comment: The conclusions of the study are simply not supported by the methods as described. Although the p values stated are significant for regular case control studies they do not meet the level of significance required for a genetic study. The discussion is tangential and should focus on a few basic points. The weaknesses of the study. The body of work that supports or refutes the study. The next step in analysis. None of these issues are directly addressed in the discussion.
Response: We have applied statistical correction for multiple comparisons and have modified the discussion to focus on the weaknesses of the study, the context within published work, and future directions.

REVIEWER 2:

Comment: Subjects section should include more information about study subjects (for example; total number, gender, age, and BMI, ethnicity, weight, smoking, hypertension, hypercholesterolemia, LDL, SBP, DBP, CAD event, drug treated etc.). Clinical characteristic and demographics of subjects may be summarized in a table. Sample details provided in the first paragraph of the results should placed in subjects.

Response: The population details provided in the first paragraph of the results is now placed in the subjects section. The total number of patients with gender, age, BMI, ethnicity, and smoking, hypertensive, hypercholesterolemia histories are now detailed in this section. All patients received a stent and therefore had a CAD event.

Comment: All clinical parameters results (given in Table 3); other than significant ones should be indicated as negative findings in results section

Response: All negative results are now specified.

Comment: Were Nominal p values given significant remaining after multiple test correction? What was the significant p value after multiple test correction? Since there were 30 clinical parameters tested against genotype, the significant p value after correction should be decreased and indicated in results.

Response: We have now detailed the Bonferroni corrected p values.

Comment: Given that there are few association studies of CAD with INSIG2 reported, the recent articles on this topic should be referred in the intro and discussed. Such as: Weidman et. all, Obesity 17, 1390–1395 (1 July 2009) | doi:10.1038/oby.2008.669 Lack of Association between a Common Polymorphism near the INSIG2 Gene and BMI, Myocardial Infarction, and Cardiovascular Risk Factors http://www.biomedcentral.com/1471-2350/10/56 Bresler J et al; which has INSIG2 genotyping within Coronary Artery Risk Development in Young Adults (CARDIA) Study and Atherosclerosis Risk in Communities (ARIC) Study cohorts

Response: We now reference these two reports.

Comment: In the discussion it is stated that there was no association found between rs566605 with CAD/MI risk factors such as obesity, T2D, hypertension, hypercholesterolemia and smoking. Such factors included in this study should be defined in the materials and methods and the findings in results section as well.

Response: These are now defined in the methods and presented in the results.
Comment: 1. In the abstract method section should be included the name of the technique used such as; RT-PCR/TaqMan/allelic discrimination.
Response: We have defined the method as suggested.

Comment: 2. In the abstract obesity risk allele which is “C” in this case needs to be added in parenthesis.
Response: We have specified the allele as suggested.

Comment: 3. Gene names should be Italicized such as; INSIG2
Response: The INSIG2 gene designation has been italicized as suggested.

Comment: 4. The version of the SDS software should be included in the genotyping section.
Response: We now include the version of the SDS software (2.01).

Comment: 5. Diagnosis criteria used (such as MONICA? Or other guideline used), and exclusion criteria from the study should be given in subjects section.
Response: Because this was a registry study there were no exclusion criteria.

Comment: 6. As there is no control sample used, this should to be declared in subject section.
Response: We now state this in the methods.

Comment: 7. Genotypic data commonly ethnicity specific and therefore Table 1 and Table 2 should be merged and stratified by ethnicity. Genotype data in different ethnicities can be compared with HapMap and NCBI dBSNP databases and should be indicated in the results.
Response: The genotype data are now stratified by race/ethnicity, presented in new tables (shown above), and compared with HapMap data.

Comment: 8. The p value after race in Table 3 needs to be clarified? (Was it for only Caucasian?)
Response: The p value indicated that the distribution of race/ethnicity was not different across genotype groups.

Comment: 9. The grant number is missing in acknowledgements (such as ;NHLBL-RO1XXXX)
Response: We now specify the grant numbers.

Comment: 10. In the discussion INSIG2 association with cholesterol should be discussed in detail. There is another study available in Korean population which can be support and should be discussed, and some supportive data in mice as well, please find below details.
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2759923/ (Association analyses of...
the INSIG2 polymorphism in the obesity and cholesterol levels of Korean populations
J Clin Endocrinol Metab. 2008 May; 93(5):1995-2001. Epub 2008 Mar 4. (A study with double-knockout mice in relation to both Insig1 and Insig2, where mice given a cholesterol-rich diet gained more weight compared to a control group)

Response: We have incorporated the relevant results from the Korean study, discuss the mouse study and reference both publications.

Comment: 11. In the third paragraph of the discussion the term "lower risk allele" is very poor use of this term. It should be clarified either the risk (C) or protective allele (G) associated with hypercholesterolemia in young adult females in predominantly white MI/CAD study cohort.
Response: We now specify the G or C alleles.