Author's response to reviews

Title: Common Genetic Variants of the Ion Channel Transient Receptor Potential Membrane Melastatin 6 and 7 (TRPM6 and TRPM7), Magnesium Intake, and Risk of Type 2 Diabetes in Women

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Author's response to reviews: see over
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Re: MS: 1269889613207236 “Common Genetic Variants of the Ion Channel Transient Receptor Potential Membrane Melastatin 6 and 7 (TRPM6 and TRPM7), Magnesium Intake, and Risk of Type 2 Diabetes in Women” Yiqing Song, Yi-Hsiang Hsu, Tianhua Niu, JoAnn E. Manson, Julie E. Buring and Simin Liu

Dear Dr. Scott Edmunds,

Thank you for the opportunity to revise and resubmit the manuscript. We appreciate the thoughtful and helpful comments provided by the reviewers. Following the instruction, we have added a methods section in our abstract to meet the journal style requirement. Now we have uploaded our revised manuscript in separate files. A detailed point-by-point response to the comments is provided below. We hope the revised manuscript meets with your final approval.

Thank you again for your time and kind consideration. I look forward to your decision.

Sincerely yours,

-Yiqing

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Referee 1: Karl Schlingmann
Reviewer’s report:
“Dear authors, you thoroughly addressed most of the points raised by both reviewers. However, I still feel uncomfortable with the statistics which are a prerequisite for your conclusions. Though important details of the nomenclature were changed, still, no absolute numbers are provided for carriers of the minor alleles of both relevant polymorphisms (V1391I and K1584E in the TRPM6 gene). I agree, that because of methodical reasons, haplotypes are inferred rather than experimentally proven, which represents a standard practice.”

Response: We thank the reviewer’s comments regarding the limitation of probabilistic estimates from our statistical haplotype inference.

“Table 3 (which remained more or less unchanged) still provides allele frequencies rather than percentages of carriers of an allele in either homozygous or heterozygous state. In contrast to table 3, you state in your Results section, that „the frequency of homozygosity for both rare alleles of these two SNPs represents 9.3% in the control samples“. A frequency of homozygosity of around 9.3% is in clear contrast to the observed haplotype frequency of the same percentage and not compatible with Hardy-Weinberg equilibrium. I assume that this percentage really represents the allele frequency rather than the rate of homozygous carriers.”

Response: We appreciate the reviewer’s pointing this out. We now have changed “homozygosity” to “carrier”. Again, we wish to clarify that our multiple locus haplotypes were inferred from phase-unknown genotype data using statistical estimation rather than experimental determination, as stated in our method, because haplotype phase cannot be deterministically estimated without any uncertainty in population studies given the existence of heterozygotes of both SNPs. It would be impossible to provide the mutually exclusive numbers of carriers of specific haplotypes. In this regard, we presented the haplotype frequencies rather than the absolute numbers for carriers.

“Therefore, the major concerns regarding the statistics remain more or less unchanged: Do you really compare carriers of the major allele with carriers of the minor allele either in heterozygous or homozygous state? At least for homozygous carriers of the minor allele an absolute number could be provided despite „phase-unknown genotype data“."

Response: We appreciate the reviewer’s comments. It is true that the absolute number for homozygous carriers of the minor alleles alone can be clearly presented. However, the absolute number for heterozygous carriers are always ambiguous and cannot be equally presented due to
“phase-unknown genotype data”. To provide informative evidence, we therefore compared “carriers” for each specific haplotype rather than “homozygous or heterozygous carriers for the minor alleles of V1393I and K1583E” in this study.

“The numbers presented in table 3 still imply a rather low percentage of homozygotes assuming Hardy-Weinberg-equilibrium. The subgroup analysis of women within the lowest quintile further decreases the number of probands in both groups (=divides them by five). You responded, that you “provided the exact numbers of individuals for our subgroup analyses for each single SNP that would allow the reader to perform crude chi-square calculations“, and refer to table 2, which however only shows allele frequencies.”

Response: We have acknowledged that we did not have sufficient sample sizes to conduct subgroup analysis stratified by the level of magnesium intake and genotypes based on either single or two SNPs. We also stated in the discussion “However, our findings could be caused by chance due to multiple comparisons and insufficient statistical power; additional studies are needed to confirm these results and to further characterize the effects of genetic variation in TRPM6 on magnesium homeostasis.” (Page 15). We did provide the exact numbers for our subgroup analyses of single SNP. Due to space limit and redundant information, we could not present both genotype frequencies and allele frequencies of all 25 SNPs in cases and controls in Table 2.
**Referee 4: Jian Zhang**

**Reviewer's report:**
“In this paper the authors investigated potential contributions of 20 SNPs in two genes called TRPM6 and TRPM7 to risk of type 2 diabetes. They suggested two SNPs that might confer susceptibility to type 2 diabetes in women with low magnesium. The paper was well written and acceptable. The statistical analysis is sounding to me in general. I have read through their point-to-point replies to the comments from the other referees. The paper has been improved.”

**Response:** We appreciate the reviewer’s positive comments.

“Discretionary revisions:
When the authors stratified all cases and controls by magnesium intake using the magnesium intake less than 250mg/day, two SNPs reach the significance threshold. The authors might need a small discussion on (robustness of this choice) how the sensitive of this 250mg/day magnesium intake.”

**Response:** The cutpoints for an exposure variable are usually prespecified according to its distribution among controls in case-control studies. As we described in the method section, we chose 250 mg/day because it was the cutpoint for the lowest quintile among controls in our study. As suggested, we rerun several sensitivity analyses by using different cutpoints for magnesium intake. We found that our results were consistent for the chosen cutpoints below 263 mg/day. Due to small sample sizes, the results tended to be unstable for any cutpoint below 232 mg/day. In this regard, we believe that 250 mg/day is a reasonable and stable cutpoint for our subgroup analyses.
Referee 3: Ching-Ti Liu
Reviewer's report:

“The authors conducted a nested case-control study among postmenopausal women in the Women’s Health Study. They analyzed 25 SNPs either in TRPM6 or TRPM7 for their association with diabetes risk. Although there wasn’t any significant association between any single SNP with diabetes risk, their sliding window haplotype analyses suggested that two non-synonymous TRPM6 variants might be the susceptibility to type 2 diabetes in women with low magnesium intake.

Major Comment
1. The authors claimed that they only consider SNP with minor allele frequency (MAF) greater than 5% in their text. However, in their result, such as table 2, they included the SNP with MAF less than 5%. For example, please explain why the SNP rs944857 was included in the study.”

Response: We appreciate the reviewer’s pointing this out. Indeed we claimed “A MAF of >=5%” as one of predefined criteria for selecting SNPs for our study. Based on the publicly accessible databases, rs944857 with the reported MAF >=5% in Caucasian populations met our inclusion criterion and was thus in our SNP list. After our genotyping work, its actual MAF tended to be below 5% in our American Caucasian women. Finally we did not exclude it in our analyses, because rs944857 is not very rare (MAF:0.09-0.2) and might carry important LD information (i.e. rs944857 is a tagging SNP and seems to have allelic frequency difference between cases and controls).

“2. On page 8, 19 window frames were used for Bonferroni correction. Please explain how to get this number with 25 (20+5) SNPs in your consideration. Shouldn’t it have 23 (19+4)? Did authors only conduct sliding window to TRPM6? If so, why not TRPM7 as well?”

Response: We indeed performed sliding window analyses for both TRPM6 (n=20 for SNP number) and TRPM7 (n=5), separately. Given the limited number of common SNPs across TRPM7 (128 kb), we did not find any positive results fro TRPM7 genotypes. At the very least, it is likely that five SNPs are not sufficient to capture the vast majority of the genetic variability of TRPM7. We have clarified this in our Discussion. (Page 13, paragraph 2).

“3. In sliding window haplotype-based analyses, the samples are stratified based on the magnesium intake. It would be better to know the results for single SNP and haplotype analyses under such stratification.”

Response: To minimize false results due to inadequate statistical power and multiple comparisons, we did not perform large numbers of subgroup analyses for all single SNP and all
possible haplotypes. Instead, we focused on those two SNPs (1393Ile and 1584Glu) with potential functionality and robust associations with diabetes in our sliding window analyses. We conducted only these predetermined subgroup analyses stratified by magnesium intake for both single coding SNPs and their derived haplotypes (please refer to our Table 3).

“4. Cross-species comparison of the protein sequences was performed among different species for TRPM6 and TRPM7. Why select different species for TRPM6 and TRPM7? Did the authors conduct the comparison over many species but only find the conserved result among the listed species?
5. In cross-species comparison of the protein sequences, three segments were selected. Please explain the motivation to select these three segments rather than others.”

Response: Regarding the cross-species comparisons, the data we presented include all the available DNA sequences from their respective species encompassing the SNP position for TRPM6 and TRPM7 in the NCBI Genbank database. Now we have added one sentence to clarify it: “…Our cross-species comparison approach for TRPM6 and TRPM7 was based solely on the availability of relevant information from the NCBI Genbank database.” (Page 8, the second last sentence).

“6. In discussion, authors mentioned their limitations on population stratification issue and claimed that majority (>92.5%) of subjects were white. Since the majority of subjects share the same ethnicity background and hence it won’t loss too many subject if exclude those non-white subjects. So it would be more convincing if authors can present the result obtained from those white subjects only too.”

Response: We appreciate the reviewer’s comments on the issue of population stratification. First of all, we would like to mention that our study population is a cohort of female health professionals throughout the US. Our matched case-control study was originally designed to follow the principles of risk-set sampling by which all cases and controls were selected from the same clearly defined source population and matched by age and ethnicity background. To avoid potential population bias, we have adopted the well-established strategy by a) matching each case-control pair on age, and ethnicity; b) performing the corresponding matched analyses; and c) performing multiple sensitivity analyses by adjusting for ethnicity variable; and d) conducting secondary analyses after excluding all nonwhite participants. Indeed we found similar results in terms the association direction and magnitudes after we excluded those nonwhite cases and matched controls, although the confidence intervals became wider unstable due to smaller sample sizes involved in our analyses, especially in our subgroup analyses. The consistency between the results from our secondary analyses and our primary results speaks to the robustness of our findings. Most importantly, it is likely that any post hoc exclusion of matching pairs after study design would substantially reduce our statistical power and compromise the generalizability of our study.
“7. In table 3, why did authors only list haplotype 0-0, 1-1, and 0-1? Where is the case for 1-0?”

Response: As we described in the Method (Page 7, paragraph 2), only haplotypes with estimated frequencies ≥ 1% in the combined cases and controls were included for analyses. The haplotype 1-0 was not listed because of its low frequency (~0.18%).

“8. In table 3, I wonder why 9.31% was presented in the text on page 10 but 9.30% was presented in the table 3?”

Response: Thanks. We have re-examined the results and changed “9.31%” to “9.3%” in the text.

“9. From table 3, it seems to me, authors only consider dominant model, which is not the same as what they did for single SNP analysis, but they didn’t spell it out in this manuscript.”

Response: To achieve relatively adequate statistical power, only dominant model was used in our subgroup analyses in Table 3. As suggested, we have indicated this in the results (Page 10, second paragraph).

“Minor Comment
10. page 4, the 5th line on the first paragraph, “28345 (71%)……. Of these 27962…” How come these two numbers are different?”

Response: Thanks for pointing this out. Indeed, 27962 of those 28345 were participants who provided baseline blood samples and were free of diabetes at baseline. We have rewritten this sentence.

“11. In the legend of Figure 2, authors placed dash line but there is not any explanation for it.”

Response: The point is well taken. Now we have explained this dash line in the legend of Figure 2.

“12. On page 11, please put the rs number for SNP K1584E too.”

Response: Thanks. We have added it.