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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Version: 2 Date: 25 August 2008

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August 25, 2008

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 considering our paper and 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 uploaded our revised manuscript in separate files. A detailed point-by-point response to each of 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

Yiqing Song, MD ScD
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“Song and co-workers report on genetic variants in two genes coding for subunits of magnesium permeable ion channels (TRPM6 and TRPM7) and their association with the risk of developing diabetes mellitus type II. …By this strategy, the authors were able to identify a haplotype of two coding SNPs within the TRPM6 gene (V1393I and K1584E; the only two non-synonymous coding SNPs with significant minor allele frequencies within the TRPM6 gene) that showed a weak but significant association with diabetes in the subgroup of participants with low magnesium intake.”

Response: We thank the reviewer’s comments noting the significance and merits of our study.

“Major revisions:
Some important aspects of the authors’ conclusions concerning the described genetic association remain unclear to the reader: As stated in the article’s text, the authors conclude that homozygous carriers of the minor-allele haplotype (1393Ile+1584Glu) had an increased risk of type 2 diabetes when they had a low magnesium intake. However, in my opinion, it remains unclear from table 3 if the authors really compared homozygous carriers of the minor allele with the remaining genotyped individuals (homozygous major allele + heterozygotes). Table 3 only lists numbers of individuals homozygous for the major allele versus heterozygotes and homozygotes of the minor allele together (i.e. GG vs. GA/AA for V1393I, AA vs. AG/GG for K1583E). For the haplotype combining both polymorphisms only allele frequencies are presented (i.e. 82.4%+7.72% vs. 9.86% for patients). The reader cannot necessarily imply the number of homozygotes from these allele frequencies of haplotypes. Therefore, the absolute numbers of carriers of the minor allele haplotype are essential and should be presented.”

Response: We appreciate Dr. Schlingmann’s comments. We now have revised our description of our haplotype results to eliminate any confusion. Specifically, we now use “carriers” for each specific haplotype including those who were either homozygous or heterozygous for the minor alleles of V1393I and K1583E. Also, we hope 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. In population genetics, statistical approaches are widely used to provide probabilistic estimates for
haplotype inference 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.

“Furthermore, assuming Hardy-Weinberg-Equilibrium, the percentage of homozygotes of the minor allele haplotype (1393Ile+1584Glu) is low (probably less than two percent). Therefore, the patients’ and control groups could hardly bear more than half a dozen subjects with homozygocity for the minor allele making reliable chi square calculations a challenge. In addition, a subgroup analysis of women within the lowest quintile of magnesium intake further significantly decreases the number of individuals per subgroup (absolute number of women within the lowest quintile for both groups not given, genotype data for this subgroup not provided). Again, percentages of allele frequencies do not reflect the absolute size of the subgroups tested.”

Response: We acknowledge 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 hope to clarify that “haplotype carriers” rather than individuals with any specific “haplotype combination” were used for our subgroup analyses. Our haplotype-based association analyses were performed using the “expectation substitution” (a.k.a. “regression substitution”) approach in which the expected haplotype dose for each subject (conditional on the estimated haplotype frequencies, observed genotypes, and inheritance model) is then treated as an observed covariate in conventionally multivariate-adjusted logistic regression. The presence of phase-unknown genotype data for haplotype inference cannot allow us to perform chi-square test. Also, crude relative risk estimates directly from chi square calculations may reflect the association trends but not provide optimal results after taking into account for confounding due to imbalance of other covariates between any two comparison groups.

“In my opinion, the reader should be able to perform chi square calculations from the numbers presented in table 3. As stated in the article’s text, the authors performed calculations under the assumption of an additive, recessive, and dominant genetic model. The results section (page 9-10) as well as table 3 only show comparisons of the major allele (wild type) with heterozygotes plus homozygotes for the minor allele (dominant model).”

Response: We have provided the exact numbers of individual for our subgroup analyses for each single SNPs that would allow the reader to perform crude chi-square calculations. As we responded to the comments above, the exact number for individuals with known haplotype combination cannot be deterministically estimated from phase-unknown genotype data using statistical estimation. We indeed have shown the main results in Table 2 for all three genetic models. To minimize false results due to small sample sizes and multiple
comparisons, we focused on dominant models for our subgroup analysis shown in Table 3. Given our study design (matched by age and ethnicity), we prefer to present the results from multivariable-adjusted models rather than univariate models or crude chi square calculations.

“Minor revisions:

page 2 “the TRPM6 gene comprises 43 exons spanning 163kb ...”
according to GenBank and Ensembl the TRPM6 gene comprises 39 exons spanning 163kb“

Response: This point is well taken. We have updated our information on the TRPM6 and TRPM7 gene structure according to the GenBank database. Now we have corrected it and cited the following reference for this sentence.


“page 2 “the TRPM6 gene” - please mark gene symbols in italics”
Response: As suggested, we have marked all the gene symbols of “TRPM6” and “TRPM7” in italics in the whole text.

“page 2 “TRPM6 is a magnesium-permeable channel protein” – TRPM6 is thought to be an ion channel subunit, if it forms homomeric channels or heteromeric channels with TRPM7 is still an unresolved issue”
Response: We have rewritten this as “TRPM6 is thought to be an ion channel subunit primarily expressed in intestinal epithelia and kidney tubules that may play an important role in intestinal and renal magnesium handling”.

“page 2 “TRPM7 ... encodes a protein of 1863 aa” – the TRPM7 protein has 1865 aa”
Response: Thanks. We have corrected it.

“page 6 “… to capture the common genetic variability across 167kb of the TRPM6 and 128kb of the TRPM7 gene, covering their corresponding 30kb 5´ upstream and 30kb 3´ downstream regions.” – did the SNPs cover 167/128kb or 167/128kb plus 60kb?”
Response: We have revised this sentence to clarify it: “… to capture the common genetic variation across the genomic regions of TRPM6 (223 kb) and TRPM7 genes (187 kb) including their corresponding 30 kb 5´ upstream and 30 kb 3´ downstream regions.”

“page 8 “the cutpoints for magnesium levels were prespecified by those for
quintiles among controls” – what was the distribution of DMII patients in the different quintiles?”

Response: The overall distribution of cases with type 2 diabetes was slightly lower than that among controls. For example, the cutpoint for the lowest quintile among cases was 242 mg/day as compared with 250 mg/day among controls. In our nested case-control study design, our population-based controls are representative of the study population and were those who would be cases if disease occurs. In this regard, the cutpoints for an exposure variable are usually prespecified according to its distribution among controls in case-control studies.

“page 9 “---based on these 20 SNPs in TRPM6 ...” – did you genotype 30 or 20 SNPs in TRPM6 (compare page 6)?”

Response: Thanks. We have corrected it.

“page 9-10 within the text and in tabel 3 you use different nomenclatures for alleles and haplotypes of the two TRPM6 polymorphisms (major allele C-T and/or A-G).”

Response: We have examined the whole text and now consistently use the A allele of rs3750425 and the G allele of rs2274924 as minor alleles, respectively.

“page 11 SIFT scores in table 4 are all <0.05 indicating “intolerant” behaviour”

Response: We have changed “Possibly affecting” to “Intolerant” in Table 4 and also indicate the classification of the SIFT scores in the footnote.

“page 11 “Given the large sizes of both genes, it seems plausible, ...” what impact has the size of the two genes ?”

Response: We appreciate the reviewer’s pointing this out. Now we have deleted this confusing sentence.

“page 12 “There are several untranslated exons in proximity to the promotor region of TRPM6 ...” – is that true?”

Response: We have changed the wording to clarify this. Now it reads “There may be some unknown causal SNPs including splicing SNPs and SNPs in the promotor region of TRPM6 that likely regulate gene expression.”

“page 13 “Given the limited number of SNPs across TRPM7 ...” – this argument is in contrast to citation 37 which you cite on page 12 -> tSNPs should be provided by the HapMap project should be sufficient to detect common haplotypes.”

Response: We have revised this sentence. “Given the limited number of genotyped SNPs (n=5) across TRPM7 (128kb) from the reference panel (HapMap database), it is likely to speculate that the genetic variability of TRPM7
may be truly limited in the general population.”

“page 14 “we lacked stable and reliable measures of extracellular or intracellular magnesium status ...” – is there information on serum magnesium values or urinary excretion?”

Response: We acknowledge that we did not measure serum or urinary magnesium levels in our study population due to specimen requirements and blood sample status.

Referee 2: Vincenzo Trischitta
Reviewer's report:
“The authors have conducted a nested case-control study of 718 postmenopausal women aimed at examining the associations between variations at TRPM6 and TRPM7 loci and the risk of type 2 diabetes (T2D). Several tagging SNPs in both genes were analyzed but no significant association was observed. However, among individuals with low magnesium intake was inadequate (<250 mg per day; i.e. in the lowest quintile of the control sample), haplotype analyses showed that carriers of both the rare alleles at two non-synonymous SNPs in TRPM6 (Val1393Ile and Lys1584Glu) were significantly more likely to be affected by T2D (i.e. to be cases, rather than controls). In addition, bioinformatics analyses indicated potential functional relevance of TRPM6 Val1393Ile SNP, thus reinforcing the biological plausibility of the observed association. The finding is novel, the design is excellent (i.e. a nested case-control based on prospective study with up to 10 years of follow-up, and an effective matching strategy used in a well-characterized population) and the paper is well written.”

Response: We appreciate Dr. Trischitta’s positive comments noting the significance of this research topic and merits of our study.

“The only severe limitation is the small sample size which does not allow the authors to detect modest genetic effects and which increases dangerously the risk of false results caused by statistical fluctuation.”

Response: As stated in the discussion, we acknowledge that our study did not have sufficient power to assess modest genetic effects and interactions and our findings could be false positives. We also emphasize this limitation in our conclusion in the abstract and the discussion that further replication in future large studies are warranted.

“A second limitation is the lack of replication in an independent population. All this is clearly stated by the authors who are, as a matter of fact, very cautious in interpreting and commenting their own data. Although I understand that nowadays studies on the genetics of complex traits are asked to be performed in (multiple) samples, which
are large enough to maximally reduce the risk of false results due to chance and/or population stratification, I still believe that “hypothesis generating by a well designed study to be further tested in subsequent attempts” is still one the most effective way to help science progressing. No matter if the hypothesis is further confirmed or not. This is, exactly, what this paper does.”

Response: We appreciate Dr. Trischitta’s comments. To the best of our knowledge, this study may provide first genetic association evidence to test the relation between common genetic variants in two major candidate genes critical for magnesium homeostasis and risk of type 2 diabetes. We agree that replication studies are required to confirm or refute these initial findings. We also hope that this study would also stimulate and support future function studies to offer new insights into the genetic basis for the homeostatic regulation of magnesium metabolism and their roles in the etiology of type 2 diabetes.

“The only minor suggestion I have is to shorten the Discussion.”
Response: As suggested, we have limited our discussion to less than 4 pages.