Author's response to reviews

Title: SLC6A3 and body mass index in the Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial

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

We thank the reviewers for their thoughtful comments. A point-by-point response follows. We have included a copy of the manuscript with the revisions underlined and in red. Page numbers are provided in the comment responses referring to these revisions. We have reworked our introduction and discussion to provide a clearer background; the majority of these changes are not underlined (as this would have involved marking a large portion of the text), but are instead referred to in the comments below.

Comments from the statistical adviser:

In general, the description of statistical analysis in the manuscript is not very clear.

We have reviewed the Methods with our statistical team and strived to improve the clarity. A specific paragraph describing the Design has been added (pages 5-6). The points raised regarding conditional logistic regression and the haplotype approach are addressed in the Methods (see details below).

I agree with reviewer E Shyong Tai that the author may need to consider using BMI as a continuous variable rather than categorical variable. In some clinic studies, people may use BMI as categorical variable for convenience. However, in the study of this manuscript, authors hope to test association between BMI (or changes of BMI) and markers. Transforming continuous BMI into categorical variable can cause loss of power.

We appreciate the reviewer’s concern regarding the potential loss in power involved in modeling the effect of BMI as an ordinal rather than a continuous variable. However, we selected this approach for 3 reasons that we consider important:
1) In the literature, BMI is virtually always classified into 4 groups: underweight, normal, overweight, and obese based on the well-established WHO categories. Using these categories assists interpretation and provides a facile comparison with other work in local, national and international settings. It provides meaningful risk estimates in these substantial and well-established categories. For example, the categories were used in an investigation of energy balance and intake in the Prostate Lung, Colon and Ovary cohort (see Chang S-C et al, CEBP 2006;15(2):334-41), the same cohort used for the present report.

2) We believe that small variations in weight are not especially meaningful. Most of the manifold health consequences of BMI are associated with these established categories, also favoring the ordinal classification.

3) As one of the other reviewers pointed out, self-report data (although quite commonly used in the literature) is subject to a degree of misclassification. This also favors classifying the data into well-established, broad and meaningful categories.

The better way is using original continuous BMI and using linear regression instead of conditional logistic regression.

The original study design focused on current and former smokers and therefore the sample included sampling according to these categories and the other covariates indicated below. We used logistic regression in a conditional analysis because the data were originally sampled by categories of current smoking status and cigarettes per day (7), age group (4), and gender (2), and conditional LR most appropriately models the sampling frame to obtain proper risk estimates. We did attempt to conduct an unconditional analysis, but including the proper terms (i.e., for each of the 56 strata) resulted in non-convergence of the model, so this approach was not tenable.

Logistic but not linear regression permits calculation of risk estimates and confidence intervals, a traditional method of depicting associations involving SNPs and phenotypes.

In the manuscript, authors mentioned "a case-control design is used". This is a little confusing. More details should be provided. It seems to me this study is not a case-control study.

The reviewer is correct. Although the original sample was based on a ‘case’ (current smokers) and ‘control’ (former smokers) sample, the present analysis by BMI is appropriately classified as cross-sectional and we have made appropriate changes in the text (page 7).

Also, if authors use conditional logistic regression, they may need to consider if it is computationally not efficient in their study and if (unconditional) logistic regression can be used.

As mentioned above, conditional logistic regression was used to ‘condition’ on the sampled variables (smoking status, smoking intensity, age group, gender). We attempted to use
unconditional logistic regression (see above); however, given the sampling involved in subject selection, we felt conditional analysis was most appropriate. The original study sampling frame was selected to evaluate smoking and BMI (see Morton et al, Pharmacogenet Genomics 2006;16(12):901-10 and Wang et al, Hum Genet 2007;122(1):41-9). Accordingly, we sampled on smoking, gender and age, assuming that the categories of BMI would be well represented. We have added information on this sampling to our Methods Design section (see earlier point above) and Statistical analysis section (page 7-8).

Reviewer 1: E Shyong Tai

Major Compulsory Revisions
1. I think it is important for the authors to better define the aims of this study. Is it to examine the associations between these polymorphism s and obesity, or is it to examine how these polymorphisms “contribute to inter-individual differences in obesity, alcohol dependence and tobacco use” If the latter, then they need better definitions of alcohol dependence. I also found the use of “former smokers” as a marker for the ability to stop smoking rather weak.

We realize that in adjusting the report for the word limit, we inadvertently dropped some explanatory information originally included in the Introduction. We have focused the Introduction and Abstract on BMI and reintroduced a succinct statement of the aims back into the Introduction (pages 2, 4). We also have altered the organization of the Results section to lead with the key findings related to BMI (page 9). Cigarette and alcohol dependence were early aims of the study design, but the number of heavy alcohol users in the PLCO cohort is limited, so we had insufficient power to address this.

We agree that ‘former’ smokers do not constitute an ideal classification; however, it is the best available in the PLCO cohort in order to identify subjects who have quit smoking, which we know is an important covariate for BMI. In fact it does show the expected relationship to BMI (see Table 1), so while we expect some degree of misclassification, use of the variable in modeling is clearly beneficial overall. We know that smokers who subsequently quit smoking gain weight, so we wanted to incorporate this in the modeling.

2. I have some concerns about including multiple ethnic groups in this study because the definition of ethnicity is relatively lax (self report with no consideration of the ethnicity of the parents) and I don’t really know what “others” is. As such, I feel that the analyses may have failed to adjust adequately for the possibility of population stratification. Given the rather small numbers of African-Americans and others, my recommendation would be to limit the analysis to Caucasians.

We understand the theoretical concern; however, we suggest that for a variety of reasons the concern is not relevant in this setting. First, published work from our own group that shows that bias from population stratification is generally modest and only becomes problematic when a stringent set of conditions are met (see for example: Counterpoint: Bias from Population Stratification Is Not a Major Threat to the Validity of Conclusions from Epidemiological Studies of Common Polymorphisms and Cancer, Sholom Wacholder, Nathaniel Rothman and Neil Caporaso, CEBP 2002;11(6):513-20). Second, and relevant to this case, adjustment by race does
not result in any change in the findings. Also, we mention that the haplotype analyses, potentially more sensitive to ethnic variation, were only performed in the Caucasian component of the sample. We specifically reran the analyses limited to non-Hispanic Caucasians. We have highlighted a statement indicating that the results were consistent in the text (page 10).

3. Perhaps the editors might consider additional statistical review on one issue. That is, whether conditional logistic regression is the most appropriate way to test for associations with obesity in this instance. I am not a statistician but I usually associate the use of this analytical method to a matched case-control design. Despite the stratified design, this is not a matched case control study as individuals were not selected on the basis of obesity. It is a cross-sectional study with stratified sampling based on sex, age (55-59, 60-64, 65-69, 70-74 years), smoking status, and quantity of cigarettes smoked for current and former smokers (1-10, 11-20, 21+ cigarettes per day). I think we need a little more detail of the manner in which the sampling was stratified. Was it carried out to ensure equal numbers in each category? Furthermore, I think it would be useful for the authors to tell us why they stratified the sampling in this way? Did they anticipate that the effects would be different in specific groups of individuals? Indeed, in the background, the authors seem to suggest (and I may be mistaken in this) that the effects of genetic variation at this locus on obesity might be seen only in smokers. If they believe that smoking modifies the effect of these polymorphisms, then they need to include an interaction term in their statistical models. They also need to demonstrate that their study was adequately powered to detect such an interaction.

Please refer to our response to the use of conditional regression and stratification of the sample in the statistical review section and below in comment #8

4. The authors need to acknowledge the limitation that BMI was self-reported. This can introduce some measurement error (particularly at the extremes of the distribution) which is non-random. This does not mean that the data is of no value. I am just suggesting that the authors mention this as a limitation and discuss the potential impact on their findings. When the BMI was assessed at age 20, 50 and baseline, did they use the same height? Or were subjects allowed to provide estimates of height at each age too. If they are using the same height, then they should realize that the percentage change in BMI is really the percentage change in weight (the height cancels out).

We agree that self-report has an inherent degree of misclassification and this is an important reason why we categorized BMI by the well-accepted WHO categories (under/normal/over/obese). One point is that PLCO collected weight information on repeated occasions and as the data was collected on > 150,000 subjects at 10 centers across the United States, the approach was highly standardized, a factor that diminishes (but does not eliminate) misclassification error. We have added a succinct treatment of this and other study limitations and the potential impact on the findings to the Discussion (page 14). Height was only determined on one occasion so the change in BMI does reflect change in weight, as the reviewer helpfully pointed out, and changes have been made to the Methods, (page 7), the Results (page 10) and the tables.
5. I am a little uncomfortable with the haplotype analysis as it has been described. The authors say that they assigned the most probable haplotypes for each individual. I am not sure that this is the most appropriate way to do this. Since these are unrelated individuals (phase unknown) and the haplotypes are estimated, it might be better to carry out the tests of association in a regression framework as has been done, but to use the posterior probabilities for each haplotype in each individual to take into account the uncertainty of the phase. Again, I think I am correct on this but I would be happy to have it pointed out to me if that is not the case. Personally, I use an R package called haplo.stats (Mayo Clinic, http://mayoresearch.mayo.edu/mayo/research/biostat/schaid.cfm) to do this.

The haplotype analyses were conducted among non-Hispanic Caucasians only. For haplotype analyses using conditional logistic regression, we considered the most probable haplotype assigned within SAS Genetics. The probability of the assigned haplotype pair was greater than 99% for approximately 80% of individuals. These points have been integrated into the Statistical analysis (page 7). Although we agree with the reviewer that we would ideally conduct our haplotype analyses using a program such as HaploStats, which accounts for phase ambiguity, such programs do not allow for conditional logistic regression modeling.

6. In relation to the genetic analysis, I think it is important that the authors tell us what model of inheritance was used to examine the associations (additive, dominant, recessive, general effects). I am not used to seeing a p-value for trend used in genetic association studies. If they believe that there is a trend from wild-type homozygotes, to heterozygotes to rare allele homozygotes, then they should just do the analysis under an additive model of inheritance and report the effect “per allele”.

Our genetic analysis was performed under an additive model of inheritance. The multivariate conditional logistic regression analysis was performed by genotype, and we report the ORs by genotype and the p value for a statistical test of a trend in the genotype ORs. This trend test is equivalent to testing under an additive model. We have added this information to the Statistical analysis Methods (page 8).

7. I actually think that the association between the SNPs and the change in BMI is important and could be moved to the main paper. However, I might only use the percentage change in BMI, and perhaps use it as a continuous, rather than a categorical variable. The description of these findings in the results section (lower portion of page 10) is difficult to understand. I think what the data shows is that individuals who carried the 9 allele for the VNTR on a wild-type background for the other SNPs, were less likely to experience large increases in weight from age20 to the time of the baseline examination. That’s really all that needs to be said.

We have moved Supplemental Table 2 into the main paper and simplified the discussion of these findings in the Results (page 10). We appreciate the reviewer’s concern to model percent change in BMI (weight) as a continuous rather than a categorical variable; however, we selected this approach for reasons similar to our categorization of BMI. First, we believe that
small variations in weight change are not especially meaningful, and second, as noted by the other reviewer, self-report data is subject to a degree of misclassification, which is minimized by selecting well-defined ordinal categories. These points favor categorizing the data to reduce this bias.

8. In the discussion, The authors state that these findings “extend our earlier findings associating polymorphisms of DRD2 and ANKK1 in relation to both smoking and obesity. Were their earlier findings based on data from the same population? If so, this needs to be stated. Even though no statistically significant interactions between these other polymorphisms and SLC6A3 polymorphisms were noted, were the effects of the SLC6A3 polymorphisms additive, and independent, of those of polymorphisms at these other loci? Was the study adequately powered to detect these interactions?

We have made changes in the manuscript to respond to this point. Specifically we highlight the earlier finding reported from this study involving the DRD2 gene in the Introduction (page 4) and the Discussion (page 11) We also clarify the relationship between these studies in the Methods (pages 5-6, 7-8).

We did attempt to explore potential interactive effects of SLC6A3 with DRD2; however, formal analysis of effect modification requires even larger study sizes than the already substantial sample we present here. Given this power limitation, we are only able to state that the analyses conducted did not suggest any hint of an interaction, and the effects of the polymorphisms were additive; this is mentioned in the Discussion (page 14).

Minor Essential Revisions
9. In the background, the authors point out that “The genetic variation that contributes to susceptibility to obesity in the general population remains unknown [3, 7].” While this was true at the time the 2 cited references were written, it is no longer true (Curr Opin Lipidol. 2008 Apr;19(2):113-21, Nat Genet. 2008 Jun;40(6):768-75).

We have cited the literature provided by the reviewer and surveyed recent GWAS and other studies to update current status of candidate modifier genes and BMI. In an attempt to clarify our study aims and the scientific basis for the study, we have rewritten a portion of the Introduction (pages 3-4) and Discussion (page 12) to include this information.

10. I am really not sure what the purpose of table 1 is. Do the authors really want to show us the other factors (other than the polymorphisms) that are associated with obesity? It does not seem to be the aim of this paper. Perhaps all that is necessary is to just show the characteristics of the study population without testing for associations between obesity an all the variables. If the authors agree, then they should also remove the first paragraph on page 9, under the heading “body mass index.”

In accordan with the reviewer’s point, we have adjusted Table 1 to no longer include p-values. However, we feel that it is important to demonstrate that, in our study sample, BMI shows the expected relationships to key variables, and have kept a portion of the first paragraph describing these findings in the text (page 9). If the Editor feels it necessary, we can cut these few sentences.
11. In the abstract, and the results, in references to the VNTR, the authors say (**as the reference). I think they actually need to state what this symbol means. I am not aware that it is a common accepted nomenclature for anything other than the common allele.

The definition of * is any allele other then the ‘9’ repeat. The definition is provided in each table where the symbol is used. However, we realize that this may not be clear in the text and have added a sentence defining the symbol in the Methods (page 6).

**Discretionary Revisions**

12. The authors may wish to consider using BMI as a continuous variable rather than categorizing individuals. The multinomial logistic regression with 4 different levels of outcome (which strict speaking should be treated as ordinal) is a little confusing and difficult to get a handle on. The authors could also consider combining the underweight and normal individuals into 1 category.

Please refer to our earlier comments to the statistician’s review that address this point.

13. In the results section, as mentioned in another comment, the authors have to decide whether the association with smoking, or in this case smoking cessation is really an aim of this project. Personally, I think it rather weakens the paper and perhaps should not be included.

Please refer to our response to Comment #1. We agree with the reviewer and limited our discussion of smoking in the Results and Discussion sections of the manuscript.

**Reviewer 2: Caroline Davis**

1. First of all, the title of the paper is misleading/inaccurate because it only refers to the SLC6A3 gene and BMI, when the results reported in the paper relate to smoking, alcohol use, and other dopamine genetic markers.

We have made changes to the manuscript to clarify the aims of the study to focus on BMI and SLC6A3. In this context, we feel that the title is no longer misleading, as it succinctly describes the main aim and findings of the paper.

2. In my view, the Introduction of the paper does not provide a suitable theoretical framework for the study, nor a compelling rationale for why the study variables are being examined, or how this investigation fits into the broader picture of dopamine and addictive behaviours. One example of this deficiency is the absence of a discussion of the functional significance of possessing the 9-repeat versus the 10-repeat of the DAT1. Some of this is dealt with in the Discussion section, but it would make a lot more sense to provide a sound theory and specific hypotheses at the beginning of the paper, not as a discussion piece.
We have introduced a new section into the Introduction to address these concerns (pages 3-4). Specifically, we have moved Discussion points regarding the DAT1 VNTR functionality to the Introduction and aimed to provide a stronger rationale and hypothesis in the Introduction. We have also added points to our conclusion to provide broader context for our finding (page 14). (Please see next point as well)

3. Further to the point above, there is no clear explanation for why dopamine is important, and why the dopamine transporter specifically. Indeed, as we later learn, other dopamine genes are also relevant, but again, we are never told why these genes are being examined, instead of a host of others.

Here are some of the points now incorporated to address the points raised by the reviewer:

SLC6A3 is perhaps the most important polymorphic gene influencing the disposition of dopamine in the synapse and mesolimbic pathway in the central nervous system. Dopamine is a key neurotransmitter mediating reward and therefore it is relevant to diverse behavioral conditions including obesity. Numerous studies have identified genes involved in dopaminergic central pathways as important in weight regulation. Among them, SLC6A3 was one of the very first genes studies in relation to smoking, alcohol intake. There is evidence for a relationship of SLC6A3 genotype to BMI in a small study in African Americans (Epstein et al, Obes Res 2002;10:1232-40) but a large, representative United States population has not been studied to date. We have added clarification to our Introduction (pages 3-4).

4. The authors fail to acknowledge the limitations of their data - in particular, that all the response variables are based on self-report information, and in many cases on retrospective recall that may have extend back 50 years. At the very least, given the emphasis on BMI, I would have liked to see measured height and weight at the time of assessment rather than data based on self-report.

Please see our earlier comments that pertain on this point (Statistician Review; Reviewer #1, Comment #4).

5. With respect to the data analyses, the authors carried out a large number of statistical tests, but they don’t seem to have adjusted for this by using a correction factor for multiple comparisons.

This was a candidate gene study based on prior hypotheses for the role of this gene involved in dopamine processing and BMI. We believe that it would be inappropriate to penalize the findings as every SNP tested had a prior hypothesis.

6. There are also several more minor, editorial issues with the paper. For example: i) in the 3rd line of Background on page 3, “make” should be “makes”; and later on, on the same page, “death of” should be “death from”; ii) on page 4, what is meant by “Smokers with high food reinforcement”? This concept needs explanation; iii) on page 9, what do the authors mean by “in a dose-dependent model”.
Thank you for identifying these points. The editorial issues have been corrected. A dose-dependent model refers to an additive model; we have clarified this in the Methods (page 8).

We hope that these changes are acceptable and look forward to hearing from you.

Sincerely yours,

Elizabeth Azzato
Neil Caporaso