Author’s response to reviews

Title: Fatty acid binding protein 3 (FABP3) is associated with insulin, lipids and cardiovascular phenotypes of the metabolic syndrome through epigenetic modifications in a Northern European family population

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Version: 5 Date: 11 January 2013

Author’s response to reviews: see over
Dear Dr. Hinney,

It is my pleasure to submit to your attention our revised manuscript entitled “Fatty acid binding protein 3 (FABP3) is associated with insulin, lipids and cardiovascular phenotypes of the metabolic syndrome through epigenetic modifications in a Northern European family population”. As you suggested, we have addressed the reviewers’ comments point-by-point with special emphasis placed on your comments. In doing so, we have revised our manuscript accordingly. We hope that you and the reviewers find the revised manuscript acceptable to be published in BMC Medical Genomics.

Please don’t hesitate to contact me for any questions related to this article.

Thank you.

Sincerely yours,

Yi Zhang
Response to Editor’s comment that is not in Reviewers’ reports:

Editor’s comment:
4) The authors need to clarify how they dealt with IRB in children.

Our response: All subjects studied were adults. We didn’t include children (<18 yr) in this project. To make it clear, we have modified the relevant description in the Methods (1st paragraph): “All study procedures for all participants were approved by the Institutional Review Boards of the Medical College of Wisconsin (HRRC#013-00).”

Reviewer’s report
Title: Fatty acid binding protein 3 (FABP3) is associated with insulin, lipids and cardiovascular phenotypes of the metabolic syndrome through epigenetic modifications in a Northern European family population
Version: 2 Date: 6 December 2012
Reviewer: David Serre

Reviewer’s report:
This is a potentially interesting study testing whether DNA methylation variation at the promoter of FABP3 is associated with metabolic syndrome phenotypes. The study design and the use of family-based approaches is novel and appropriate for the question investigated. I especially appreciated the inclusion of heritability analyses.

Major Compulsory Revisions
1- The authors perform a large number of tests but seem to correct only for the number of CpG tested (N=17 or 18), not for the number of phenotypes (N=42) (page 13). The actual number of tests performed is at least 714 (42*17) and needs to be corrected for.

Response to reviewer: In our study, we performed a methylation association analysis of each of our measured MetS phenotypes. We have evaluated each of these phenotypes as a separate hypothesis. A similar approach has been repeatedly used in genetic studies addressing multiple traits of a disease, for example, Fox et al. conducted a GWAS study on several traits reflecting fat distribution and corrected only for the number of SNPs tested for each trait separately (PLoS Genet. 2012;8(5):e1002695). We do agree with reviewer’s perspective on this that since many of MetS traits might be intrinsically related and we yet don’t have a way to assess which traits are related and which traits are separate, it might be helpful to provide the conservatively corrected p-value for readers’ reference. We have added this information and the rationale in the Results (the fourth paragraph).

2- The effect sizes observed are very small (<2%) and it is not obvious to me that these are meaningful clinically or biologically. This should be more extensively discussed (displaying sex average would for example help understanding whether methylation levels are indeed "strongly affected by sex"). The authors should also try to refrain to use "strong" and other adjectives to qualify these correlations.
**Response to reviewer:** In our study, we have found that the variation in a Mets phenotype that can be attributed to individual CpG site methylation is comparable to the estimated effect sizes of individual SNPs upon similar traits in previous studies. This suggests that in this complex disease model, multiple genomic DNA methylation variations contribute in a synergistic manner similar to that observed for SNP variants. That might be the reason we observe that each individual site contributes to only a small portion of the total phenotype variance. Clinically however, this is meaningful because even small changes in mean levels of various risk factors have a measurable benefit at a population wide level. Most intervention trials using diet and drugs report mean alterations comparable to those seen in our population, but these are associated with significant change in the incidence of cardiovascular and other diseases. We have added these statements in the Discussion and the relevant articles have been added to the References. As for the effect of age and sex, we have modified the relevant part in the Results based on Reviewer’s suggestions.

**Minor Essential Revisions**

3- it is not clear whether all 517 individuals were assessed for DNA methylation and if this was done in triplicates (page 7). If multiple replicates were performed, how similar were they?

**Response to reviewer:** Yes all subjects were being assessed in triplicates. We have rejected the entire methylation datasets of 13 subjects (2.5%) with calling rate lower than 95%. Of the remaining subjects, we excluded measurements that have standard deviation higher than 0.1 (4.8% of all measurements are excluded). We have added this information in the Methods.

4- the authors state in the abstract and introduction (page 5) that FABP3 "transcript levels in PWBCs are correlated with MetS leading components". They never use these data in their study. I would like to see some correlations between FABP3 methylation and gene expression (which would be much more convincing that the EMSA experiment presented).

**Response to reviewer:** Based on the comments from both reviewers, we have decided to add a new table showing the correlation results of FABP3 gene expression assayed in PWBCs with measures of MetS in our study cohort. This information is presented in the new Table 4. We also agree with both reviewers that the evidence shown in the EMSA experiments are less informative than the correlation results on FABP3 gene expression. Therefore we have removed the description of these experiments. Since the gene expression analysis on MetS phenotypes has been performed on a different cohort of subjects (in whom only clinical outcome phenotypes were measured) than the current cohort of subjects for whom we have measured the FABP3 promoter methylation levels, we didn’t find an overlap cohort with which we could conduct the correlation tests on methylation and gene expression of FABP3. We thus thought that the evidence presented in Table 4 will be an alternative way of linking regulation of FABP3 gene expression by promoter methylation with the expression of MetS phenotypes in our subjects. Please see the Results (fifth paragraph) and Discussion (sixth paragraph).
Discretionary Revisions

5- claiming that peripheral blood DNA methylation is highly correlated with that of other tissues is a bit shaky given the amount of data emphasizing tissue-specific methylation patterns. Why not directly show from the heart tissue used in the EMSA experiment that the pattern of methylation at the FABP3 promoter are similar (or not) in heart and PWBC?

Response to reviewer: We have modified and expanded our discussion about conducting a methylation study using peripheral blood in the Discussion (the first paragraph). Reviewer has suggested a good experiment to further address the tissue-specificity of FABP3 methylation. We think this can be included in our future study on the function of this gene.

Level of interest: An article whose findings are important to those with closely related research interests
Quality of written English: Acceptable
Statistical review: Yes, and I have assessed the statistics in my report.
Declaration of competing interests: No competing interests
Reviewer's report
Title: Fatty acid binding protein 3 (FABP3) is associated with insulin, lipids and cardiovascular phenotypes of the metabolic syndrome through epigenetic modifications in a Northern European family population
Version: 2 Date: 14 December 2012
Reviewer: Reid S Alisch
Reviewer's report:

Major Compulsory Revisions:
1. It needs to be clear, at least in your discussion, how to interpret your EMSA data in light of the fact that MetS patients have elevated levels of HFABP. Since the "completely methylated" promoter is not physiologically relevant, as only 2/17 CpG units interrogated were near 100% methylation, while most others 14/15 were less than 40% methylated, perhaps a simple reporter/expression vector system will provide a more thorough dissection of the promoter, especially needed for the claims made by the authors here. Hence, it would be more relevant if you could compare the expression levels of a promoter with 100% methylation at CpG units 20 and 21/22 to a promoter with 0% methylation at those same two sites.

Response to reviewer: Our EMSA experiments were originally designed for testing the two extreme states of 0% methylation (hypothetically open) vs. 100% methylation (hypothetically closed) of FABP3 methylation. We agree with this reviewer that this might have created a methylation state that is not physiologically relevant. We also agree with both reviewers that these EMSA results are less informative than showing the relevance with gene expression of FABP3 in our cohort. We therefore have removed the description of our EMSA experiments from the manuscript. In combining the comments of both reviewers, we thought it might be better if we present data of FABP3 gene expression in correlation with MetS phenotypes instead. This data is summarized in the new Table 4. Please see the Results (fifth paragraph) and Discussion (sixth paragraph).

Minor Essential Revisions:
1. Authors need to define the definition of CpG island shores that they are using for this manuscript, as it doesn't meet the conventional of 2000 nucleotides on each side of a CpG island Irizarry et al. 2009).

Response to reviewer: To comply with the more commonly accepted system as the review referred to, we have modified our statement regarding locations of the CpG sites included in our analysis. Please see in the Results (the second paragraph) and in the figure legend of Figure 1.

2. Please state the sensitivity of the assay as many of the differences found are <2%.

Response to reviewer: We have further addressed the sensitivity/repeatability question in the Methods. “All subjects were being assessed in triplicates. We have rejected entire methylation datasets of 13 subjects (2.5%) with calling rate lower than
95%. Of the remaining subjects, we excluded measurements that have standard deviation higher than 0.1 (4.8% of all measurements are excluded).” We are unclear about the <2% differences that Reviewer referred to. If that was referred to the effect sizes of methylation on phenotypic variance, please see our response here: In our study, we have found that the variation in a Mets phenotype that can be contributable to individual CpG site methylation is comparable to the estimated effect sizes of an individual SNP to a similar trait from previous studies. This suggests that in this complex disease model, genomic DNA methylation variation contribute in a similar synergistic way as SNP variants do. That might be the reason that we observe individual site contributes to only a small portion of the total phenotype variance. Clinically however, this is meaningful because the changes in mean levels of various risk factors are usually small, but even small changes in mean levels can have a measurable benefit at a population wide level. Most intervention trials using diet and drugs report mean alterations comparable to those seen in our population, but these are associated with significant decline in the incidence of cardiovascular and other diseases. We have added these statements in the Discussion as well as relevant articles to the References.

3. Define CpG island shores. Irizarry et al. 2009 referred to them as 2000 bp on each side of a CpG island. This definition doesn’t seem to fit your description.

**Response to reviewer:** We have addressed this in Results (the 2nd paragraph) and in the figure legend.

4. Combining CpGs as one “unit” may provide a misleading level of methylation at either CpG.

**Response to reviewer:** Limited by the Mass-spec method implemented in the EpiTYPER® assay, for 9 out of 22 individual CpG sites, we can only measure their quantitative variation in methylation in groups of adjacent CpG sites, as their genomic positions are too close for the Mass-spec to call as individual fragments. This is a technological limitation inherent in all EpiTYPER results, which require cautious interpretation when it comes to sites that have to be called as groups (or units). We have acknowledged this in the Discussion.

5. In the discussion the authors state: "High levels of correlation in CpG methylation profiles between peripheral blood and other tissue types..." This is overstated and should be modified as CpG methylation is highly tissue type specific.

**Response to reviewer:** We have taken reviewer’s advice and had revised our statement about the tissue specificity of CpG methylation. We further expanded the discussion on why we believed using peripheral blood for CpG methylation study for MetS is a valid approach. These modifications are in the first paragraph of the Discussion.

**Level of interest:** An article of limited interest

**Quality of written English:** Needs some language corrections before being Published
Response to reviewer: We have gone over the entire manuscript and made corrections where we think the language can be improved.

Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.

Declaration of competing interests: I have not competing interests.