Author's response to reviews

Title: Genetic variation of Glucose Transporter-1 (GLUT1) and albuminuria in 10,278 European Americans and African Americans: a case-control study in the Atherosclerosis Risk in Communities (ARIC) Study

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Author's response to reviews: see over
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Genetic variation of Glucose Transporter-1 (GLUT1) and nephropathy in 10,278 Caucasian and
African-Americans: a case-control study in the Atherosclerosis Risk in Communities (ARIC) Study

Dear Dr. Zauner:

Thank you very much for considering our manuscript titled “Genetic variation of Glucose
Transporter-1 (GLUT1) and nephropathy in 10,278 Caucasian and African-Americans: a case-
control study in the Atherosclerosis Risk in Communities (ARIC) Study”.

My co-authors and I would like to submit a revised version of our manuscript for your
consideration. We appreciate the suggestions from the reviewers and have attempted to address
each of the comments (see attached).

The revised manuscript with the changes made as a result of these reviews has been attached.

Thank you. Please do not hesitate to contact us if there are any further questions.

Sincerely,

Josef Coresh, M.D. Ph.D. Charles Hsu, M.D. Ph.D.
Reviewer Shiro Maeda:
Hsu et al. examined the association of SNPs in GLUT1 with albuminuria in 8,122 European American subjects and in 2,156 African American subjects. They selected 6 SNPs for the stage 1 analysis, and identified modest association of rs841847 (Enh2) with albuminuria (micro + macro) in European American subjects with type 2 diabetes, but not in subjects without diabetes. Then they selected this SNP for further analysis (stage 2), and the authors could observe the same trend of the association of this SNP with albuminuria in European subjects with type 2 diabetes. They further demonstrated that the effect of Enh2 might be interacted with elevated plasma insulin concentration in European American subjects without diabetes. The authors could not identify any effects of the GLUT1 SNPs on albuminuria in the African American population. The authors have performed important attempt, and provided interesting information, but there are several concerns in the present form.

MAJOR COMMENTS:
1) Although the authors reported that the rs841847 (Enh2) was significantly associated with albuminuria in European subjects with type 2 diabetes, the number of subjects with type 2 diabetes was too small to lead correct conclusion.

RESPONSE:
Despite the fact that we had n=1095 European American patients with type 2 diabetes, I would agree that the size of our study sample is somewhat limited, particularly given the rarity of the Enh2 TT risk genotype in this population (6.6%). For this reason, we have amended the discussion of limitations to reflect this. Particularly, a separate larger study of type 2 diabetic European Americans may better evaluate the role of GLUT1 and albuminuria.

Edits:
(addition in abstract):
…the GLUT1 Enh2 risk genotype, instead of *XbaI*, may be recessively associated with type 2 diabetic albuminuria among European Americans, **though an association is not conclusive…**

(addition in discussion):
Additionally, despite the fact that our study included 8122 European Americans, of whom there were 1095 type 2 diabetics, given the rarity of the Enh2 TT risk genotype among diabetics (6.6%), the size of our study precludes any definitive conclusions regarding the association of type 2 diabetic albuminuria and the Enh2 polymorphism of GLUT1. Given this limitation, our study can at best suggest an association between Enh2 and albuminuria among European American type 2 diabetics; however, due to the limitation in our number of subjects, we do not have sufficient power to make conclusive statements regarding this association. Perhaps a larger study of type 2 diabetic European Americans involving multiple cohorts may provide more power to definitively evaluate the role of the Enh2 risk genotype of GLUT1 and albuminuria.

2) In addition, because the authors examined 6 SNPs using several models, the authors need to perform a correction of multiple testing errors with an appropriate way; the
present associations seem not to overcome Bonferroni’s correction that is considered too conservative for the present analysis.

RESPONSE:
There was strong prior evidence for Enh2 (rs841847) as a functional candidate polymorphism, both in human and laboratory studies [1-8]. Based on this *a priori* determination of the status of Enh2 (rs841847) as a functional candidate SNP, we believed it was appropriate to keep the alpha for statistical significance of p=0.05 for analyses focusing on Enh2 (rs841847). However, given that the other 5 SNPs (rs841853, rs841839, rs3768043, rs2297977, rs841858) examined were largely meant to be tagging SNPs without any prior evidence to suggest function, we felt it was appropriate for the analyses involving these to utilize a Bonferroni correction, with the adjusted alpha for statistical significance determined by dividing the significance level by the number of tagging SNPs, with adjusted p-value at the 0.05 significance level of p=0.01. For haplotype analyses, a Bonferroni correction was also applied by dividing the significance level (0.05) by the number of major haplotypes (frequency >5.0%, n=4 for European Americans, n=8 for African Americans) for haplotype-based association analysis (haplotype analysis Bonferroni corrected level of statistical significance for European Americans p<0.0125, for African-Americans p<0.00625). For diplotype analyses for European Americans, a Bonferroni correction was also applied by dividing the significance level by the number of diplotypes (n=10) comprised of the major haplotypes, with adjusted level of statistical significance p<0.005.

Edits:
Methods:

“*P* values <0.05 were considered statistically significant.” changed to

There was strong prior evidence for Enh2 (rs841847) as a functional candidate polymorphism, both in human and laboratory studies [1-8]. Based on this *a priori* determination of the status of Enh2 (rs841847) as a functional candidate SNP, we believed it was appropriate to keep the alpha for statistical significance of p<0.05 for analyses focusing on Enh2 (rs841847) with further correction. However, given that the other 5 SNPs (rs841853, rs841839, rs3768043, rs2297977, rs841858) examined were largely meant to be tagging SNPs without any prior evidence to suggest function, we felt it necessary to correct for multiple testing errors by applying a Bonferroni correction[9], with the corrected alpha for statistical significance at the 0.05 level determined by dividing the significance level by the number of tagging SNPs, with adjusted p-value<0.01.

Addition to paragraph describing haplotype analysis in Methods:

For haplotype analyses, a Bonferroni correction was also applied by dividing the significance level (0.05) by the number of major haplotypes (n=4 for European Americans, n=8 for African Americans) for haplotype-based association analysis (Bonferroni corrected level of statistical significance for European Americans p<0.0125, for African-Americans p<0.00625). Associations with albuminuria for each diplotype (of major haplotypes) were examined separately using logistic regression. For diplotype
analyses for European Americans, a Bonferroni correction was also applied by dividing the significance level by the number of diplotypes (n=10) comprised of the major haplotypes, with adjusted level of statistical significance p<0.005.

Results:

Figure 1 and legend modified to indicate Bonferroni adjustment. Addition in legend:
None of the tagging SNPs were able to reach a Bonferroni adjusted level of statistical significance. \(^1\)Bonferroni corrected level of statistical significance p=0.01

Tables 2 and 3 have been notated with the appropriate Bonferroni corrected levels for statistical significance.

Addition to results text:

XbaI and other tagging SNPs did not approach the Bonferroni corrected level of statistical significance of p<0.01.

Similarly, the XbaI risk genotype (AA) had a larger association with albuminuria among those with type 2 diabetes rather than non-diabetics (OR 2.23 [P=0.064] and 1.75 [P=0.212], respectively), though associations were not statistically significant for XbaI and did not approach the Bonferroni corrected level of statistical significance (p<0.01) (Table 2).

For the four remaining SNPs, genotypic ORs ranged from 0.72 to 1.85 (all p-values>0.17) with none of the SNPs approaching the Bonferroni adjusted statistical significance level of p<0.01.

When the ten diplotypes associated with the major haplotypes A, B, C, and D (table 3) were examined, only the BD diplotype was associated with albuminuria (OR 3.03, 95% CI 1.36 - 6.79, p=0.007, compared to individuals without the BD diplotype). However, this approached but did not reach a Bonferroni corrected level of statistical significance of p<0.005.

Overall, haplotype diplotypes homozygous for the Enh2 T allele had an increased risk of albuminuria compared to all others combined (P=0.014), though it did not reach the Bonferroni corrected level of statistical significance of p<0.005.

3) Classification of albuminuria was based on single measurements of ACR in the present study. This may be leading incorrect conclusions in case that an association between variations and phenotypes exhibits a borderline value as shown in the present study.

RESPONSE:
We agree that a single measurement of ACR could definitely lead to incorrect conclusions, particularly with potential misclassification of borderline values with a dichotomous outcome. We have included this in our limitations in our discussion. However, in order to address this issue, we attempted to do further sensitivity analyses with a more stringent outcome, macroalbuminuria as discussed previously. As seen in table 4, among type 2 diabetic European Americans, the Enh2 risk genotype appears to have an increased association with macroalbuminuria (OR 2.69), consistent with our results with albuminuria and microalbuminuria.

Edits:
(addition in discussion)

Its design is cross-sectional and the albuminuria classification is based on a spot urine ACR because urine which was only collected at one ARIC visit. A phenotype based on a single measurement of ACR could definitely lead to incorrect conclusions, particularly with potential misclassification of borderline values with a dichotomous outcome such as albuminuria. However, sensitivity analyses for macroalbuminuria demonstrated a significant risk association with the Enh2 risk genotype.

4) The interactive effect of rs841847 (Enh2) with insulin sensitivity (plasma insulin concentrations) on albuminuria has considerable interest, but it is not clear why the authors exclude subjects with microalbuminuria from the analysis.

RESPONSE:
We initially decided to exclude those patients with microalbuminuria in order to examine a more stringent phenotype. However, we believe it is important to first report associations with albuminuria, and then to focus on a more specific outcome, such as macroalbuminuria. These results are now included.

Edits:
(change and addition in Results)

To examine effects of insulin concentration on the association of GLUT1 Enh2 genotypes and albuminuria, we stratified by insulin concentrations excluding those with diabetes (to avoid confounding due to insulin treatment for diabetes). Due to the low frequency of the GLUT1 Enh2 risk genotype among African Americans, analysis was limited to European Americans. Among European Americans, (n=6583 controls, 294 cases of microalbuminuria, and 46 cases of macroalbuminuria), the mean fasting insulin concentration was 10.9 µU/mL (S.D.= 7.5). Risk of albuminuria for Enh2 TT was OR 1.08 (95% CI: 0.70 - 1.67, p=0.724), adjusting for age, gender, hypertension status, BMI, and GFR. Among individuals with insulin in the highest quartile (mean insulin 19.9 µU/mL, S.D.=9.7), the adjusted OR of albuminuria for Enh2 TT was 1.25 (95% CI: 0.60 - 2.58, p=0.547). Among individuals with insulin concentrations in the lower three quartiles (mean 7.9 µU/mL, S.D.= 2.9), the adjusted OR of albuminuria was 1.00 (95% CI: 0.58 - 1.72, p=0.990) for Enh2 TT carriers. When we focused on the more specific outcome of macroalbuminuria as a phenotype and excluded patients with
microalbuminuria, risk of macroalbuminuria for Enh2 TT was OR 1.84 (95% CI: 0.71 - 4.77, p=0.210) as illustrated in figure 2. Among individuals with insulin in the highest quartile, the adjusted OR for Enh2 TT was 4.08 (95% CI: 1.06 - 15.61, p=0.040). Among individuals with insulin concentrations in the lower three quartiles, the adjusted OR of macroalbuminuria was 1.00 (95% CI: 0.23 - 4.28; P=0.995) for Enh2 TT carriers. Among non-diabetics, a formal test of interaction of the GLUT1 Enh2 risk genotype and the upper quartile of insulin was not significant for macroalbuminuria (P=0.163).

5) I recommend the authors to examine the association of GLUT1 SNPs with quantitative traits related to renal function (e.g, ACR, s-Cr, BUN) in the ARIC population.

RESPONSE:
Given the potential association between Enh2 (rs841847) and albuminuria, we have also examined potential associations using multivariate linear regression with log-transformed ACR (data log-transformed due to the skewed distribution) and also serum creatinine among European Americans, stratified by type 2 diabetes (see below). We did not perform this analysis among African Americans given the low frequency of the Enh2 risk genotype among this population.

Edits:

Addition to Methods:
Multivariate linear regression was used to assess associations between the Enh2 risk genotype with log-transformed ACR µg/mg (data log-transformed due to the skewed distribution) and also serum creatinine among European Americans, stratified by type 2 diabetes; these analyses were not performed among African Americans due to the rarity of the Enh2 risk genotype in that population.

Addition to Results:
Additionally, among European Americans, we also examined potential associations using multivariate linear regression with log-transformed ACR µg/mg (data log-transformed due to the skewed distribution) and also serum creatinine among European Americans, stratified by type 2 diabetes (see Table 5). Though the Enh2 risk genotype tended to be positively associated with worse ACR and higher serum creatinine only among type 2 diabetic European Americans, there were no statistically significant associations. We did not perform this analysis among African Americans given the low frequency of the Enh2 risk genotype among this population.
Table 5. Unadjusted and multivariate linear regression of ln (ACR) and serum creatinine for Enh2 in all genotyped European Americans, by type 2 diabetes status.

Regression coefficient for GLUT1 Enh2 Risk Genotype(TT) (95% CI)

<table>
<thead>
<tr>
<th>(N= Enh2 TT Genotype/ All Genotypes)</th>
<th>ln (ACR)</th>
<th>Serum Creatinine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β Coefficient (95% CI)</td>
<td>P-value</td>
</tr>
<tr>
<td>TYPE 2 DIABETES (N= 72 / 1,095)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unadjusted</td>
<td>0.26 (-0.17 - 0.69)</td>
<td>0.240</td>
</tr>
<tr>
<td>Model 1</td>
<td>0.27 (-0.14 - 0.68)</td>
<td>0.196</td>
</tr>
<tr>
<td>Model 2</td>
<td>0.28 (-0.13 - 0.68)</td>
<td>0.185</td>
</tr>
<tr>
<td>NON-DIABETIC (N= 478 / 7,027)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unadjusted</td>
<td>-0.02 (-0.12 - 0.09)</td>
<td>0.737</td>
</tr>
<tr>
<td>Model 1</td>
<td>-0.02 (-0.12 - 0.08)</td>
<td>0.702</td>
</tr>
<tr>
<td>Model 2</td>
<td>-0.02 (-0.12 - 0.08)</td>
<td>0.667</td>
</tr>
</tbody>
</table>

Model 1 includes age, sex, systolic blood pressure, diastolic blood pressure and hypertension medication use.
Model 2 includes Model 1 covariates and BMI and estimated glomerular filtration rate for the outcome ln (ACR). For the outcome of serum creatinine, model 2 includes Model 1 covariates and BMI.

MINOR COMMENTS:

6) Because white population is not exactly the same as Caucasian, the description needs to be unified.

RESPONSE: We have changed our text to reflect this. We have used the term European American instead of white or Caucasian in order to clarify this ethnic description.

Edits: Please see title, abstract, text, figures to reflect this change.

7) The description for unit of plasma insulin concentration (in the results section; mg/dl) is probably wrong.

RESPONSE: Thank you very much. That was an error. It should be micro-units per mL (µU/mL). This has been corrected in the text.

Edits: Please see Results section for corrections.
Level of interest: An article of importance in its field
Quality of written English: Acceptable
Statistical review: Yes, and I have assessed the statistics in my report.
Reviewer Samy HADJADJ:
C. Hsu et al, have examined the relationship between GLUT1 genetic variations and albuminuria in a large sample of Caucasian and African-American people. The study is interesting because, as mentioned by the authors, it is the largest examination to date of the GLUT1 genetic variations according to albuminuria.

Major comments:
1) In the title and along the manuscript, the authors refer to nephropathy. In fact, they should mention albuminuria. Nephropathy is not certain in non diabetic microalbuminuric patients.

RESPONSE: We agree that it is important to utilize albuminuria instead of nephropathy, particularly in the case of non-diabetic patients. We have changed the title, abstract, and text to reflect this. Particularly, in reference to our specific study and our outcomes, we specify albuminuria. However, in our manuscript we do continue to use the term “nephropathy” to refer to the pathologic process and also in reference prior studies which have utilized nephropathy as an outcome.

Edits: Please see changes in title, abstract, text, and legends.

2) The abstract is not so self comprehensive. Indeed, the notion of stage 1 and stage 2 are clearer in the text but are not very easy to understand in the abstract. Thus, a more appropriate term as primary sample and replication sample could be used.

RESPONSE: We agree that the wording needed clarification in the abstract. We have changed the abstract accordingly.

Edits:
We conducted a two-stage case-control study nested in a prospective cohort study of 2156 African Americans and 8122 European Americans with urinary albumin-to-creatinine ratio (ACR) from 4 U.S. communities, by race and type 2 diabetes…The evaluation phase (stage 1, n=2938) tested associations of albuminuria (n=305) with six GLUT1 SNPs: promoter SNP(rs841839), intron-1 SNP1(rs3768043), intron-1 SNP2 (rs2297977), Enh2(rs841847), XbaI(rs841853), and intron-2 SNP(rs841858). Enh2 was examined in a separate sample in the replication phase (stage 2, n=7340) and in a total combined sample (n=10,278), with all analyses stratified by race.

3) The greatest concern I have is that the genotypic frequencies among Caucasians or African-Americans, are very different as seen in Figure 1. It is thus very complicated to consider both ethnicities all together.

RESPONSE: We agree that it would not be appropriate to consider both ethnicities together. In order to emphasize this, we have clarified further that all analyses were race-stratified.
Edits:
Abstract methods:
We conducted a two-stage case-control study …by race and type 2 diabetes…. with all analyses stratified by race.

Text body methods:
Because allele frequencies differed by race, analyses were race-stratified.

4) Although diabetes duration is not available, it is clearly unexpected to have only 6 proteinuric patients among 1095 type 2 diabetes people. Thus, a selection bias is likely and makes the conclusions on type 2 diabetes and nephropathy very questionable.

RESPONSE: Among the 1095 type 2 diabetic European Americans, there are 53 cases of macroalbuminuria, of whom 6 have the risk genotype. We agree that the rarity of the risk genotype can make the estimates unstable. For that reason, we felt it important to provide readers with both the number of cases with the risk genotype / the total number of cases. This was likely due to our not adequately labeling table 4.

Edits: We have corrected this by adding the label “(N Cases = Cases with Enh2 TT Genotype/All Cases)” under each albuminuria outcome.

Minor comments:
5) In the “GLUT1 Enh2 Risk Genotype and Insulin” section, the authors explained that the excluded people with microalbuminuria. This is not clearly stated why they made such a choice. It could be suggested to present the data with albuminuria first and then with only macroalbuminuric patients.

RESPONSE: Please refer to our response to Dr. Maeda’s comment #4 above.

6) The discussion is quite long and could be shorten. In the discussion, the speculations about the Enh2 SNP effects are not very clear to me. Two alternate speculations are indeed presented: - high insulin is associated with high intracellular glucose concentrations, leading to glomerulosclerosis. - the alternate hypothesis is that insulin in podocytes prevents albuminuria via GLUT1. It is not very easy to me to reconcile both hypotheses.

RESPONSE: We have clarified the text in the discussion. We did not intend to suggest that the insulin response of podocytes results in prevention of albuminuria. Rather, we meant that the insulin response of podocytes resulting in increased glucose uptake is mediated via GLUT1, and that the interaction of these factors (increased intracellular glucose, increased insulin, upregulated GLUT1) would result in worsening function of podocytes with subsequent albuminuria. We have amended the discussion to reflect this.

Edits in discussion:
Additionally, the interaction of the Enh2 SNP and insulin supports the previously posited hypothesis[5] that in individuals with the Enh2 risk genotype, high intracellular glucose concentrations might increase in mesangial cells in response to insulin[36]. The high concentrations of intracellular glucose may contribute to mesangial matrix expansion and glomerulosclerosis through several pathologic cellular mechanisms including the polyol pathway, activation of protein kinase C, increased formation of advanced glycation end-products, and the hexosamine pathway[37,38]. Additionally, there is evidence that podocytes are critical in maintaining the glomerular filtration barrier of the kidney and preventing albuminuria [10-12], and a recent study demonstrated that the glucose uptake of podocytes are insulin responsive and act via GLUT1, suggesting the insulin sensitivity of human podocytes resulting in urinary protein loss may act via these mechanisms [39].

7) The data on estimated GFR according to MDRD formula should be added in the tables, as they are cited in the method section.

RESPONSE: We agree that estimated GFR should be included in the table for baseline characteristics.

Edits: Please see table 1.

Level of interest: An article of importance in its field
Quality of written English: Acceptable
Statistical review: Yes, and I have assessed the statistics in my report.

Reference List


