Association of adiponectin gene polymorphisms with type 2 diabetes in an African American population enriched for nephropathy

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ABSTRACT

Objective: Polymorphisms in the adiponectin gene (ADIPOQ) have been associated with type 2 diabetes and diabetic nephropathy in type 1 diabetes, in mostly European-derived populations.

Research Design and Methods: A comprehensive association analysis of 24 SNPs in the adiponectin gene was performed for type 2 diabetes and diabetic nephropathy in African Americans.

Results: The minor allele (A) in a single SNP in intron 1, rs182052, was associated with diabetic nephropathy (P=0.0015, OR=1.37, CI=1.13-1.67, dominant model) in an African American sample of 851 cases with diabetic nephropathy and 871 non-diabetic controls in analyses incorporating adjustment for varying levels of racial admixture. This association remained significant after adjustment of the data for BMI, age, and gender (P=0.0013 – 0.0004). We further tested this SNP for association with longstanding type 2 diabetes without nephropathy (n=317), and evidence of association was also significant (P=0.0054, OR=1.46, CI=1.12-1.91, dominant model) when compared to the same set of 871 non-diabetic controls. Combining the type 2 diabetes and diabetic nephropathy samples into a single group of cases (n=1168) resulted in the most significant evidence of association (P=0.0003, OR=1.40, CI=1.17-1.67, dominant model). Association tests between age at onset of type 2 diabetes and the rs182052 genotypes also revealed significant association between the presence of the minor allele (A/A or A/G) and earlier onset of type 2 diabetes.

Conclusions: The SNP rs182052 in intron 1 of the adiponectin gene is associated with type 2 diabetes in African Americans.
Type 2 diabetes and diabetic nephropathy are more prevalent among African Americans than European Americans, even when taking into consideration ethnic differences in socioeconomic status, prevalence and severity of hypertension, and access to adequate healthcare(1-3). Studies of African American families with type 2 diabetes(4) or diabetic nephropathy(5) have revealed clustering of both diseases, indicating a genetic component to susceptibility. Genome scans in families have supported a genetic contribution to susceptibility to type 2 diabetes and diabetic nephropathy in African Americans(4; 6).

Plasma adiponectin levels are inversely correlated with diabetes, and insulin resistance (7; 8). In contrast, plasma adiponectin has been shown to be increased in patients with kidney disease (9) and studies suggest that increased adiponectin concentration is a predictor of subsequent kidney disease(10).

Adiponectin gene (ADIPOQ) polymorphisms have been implicated in type 2 diabetes(11) and type 1 diabetic nephropathy(12; 13). Few studies have addressed genetic variants in adiponectin and association with diabetes in Africans(14) or African Americans(15). This second report in African Americans noted several differences between European-derived samples and African Americans regarding associations between ADIPOQ polymorphisms and body composition and lipid phenotypes highlighting potential ethnic differences in the adiponectin gene and the importance of investigating variants in this gene in African Americans.

Given the paucity of studies on adiponectin gene polymorphisms and type 2 diabetes or diabetic nephropathy in African Americans and the high risk of these diseases in this population, a thorough interrogation of this gene in African Americans was warranted. We tested 24 single nucleotide polymorphisms (SNPs) in the adiponectin gene for association with type 2 diabetes and diabetic nephropathy in a large collection of African Americans residing in the southeastern United States.

RESEARCH DESIGN AND METHODS

Subjects. Cases included 851 unrelated African Americans with type 2 diabetes-associated ESRD from dialysis centers in Winston-Salem, Greensboro, and Hickory, North Carolina. All diabetic nephropathy cases had ESRD and were on dialysis at the time of recruitment. Diabetes was considered to be the primary cause of nephropathy if subjects developed diabetes after the age of 35 and diabetes was present more than 5 years before initiation of renal replacement therapy, and/or in the presence of diabetic retinopathy or proteinuria exceeding 500mg/24 hours. An additional 317 unrelated African Americans with type 2 diabetes and no renal disease, and 871 non-diabetic, unrelated controls were recruited from medical clinics, churches and health fairs in North Carolina. Diabetic subjects lacking nephropathy had diabetes for more than 10 years with a spot urine albumin:creatinine ratio <30mg/g and serum creatinine concentration <1.5mg/dl in men or <1.3mg/dl in women. Individuals were actively receiving treatment with oral hypoglycemic agents and/or insulin. Non-diabetic controls were self-reported African Americans born in the southeast, age ≥18 years, who denied a personal history of diabetes or a
personal or family history of kidney disease in 1st degree relatives. Each participant provided 40ml of blood for DNA isolation. DNA was isolated using an AutoPure LS automated DNA extraction robot (Gentra Systems, Minneapolis, MN). Recruitment and sample collection procedures were approved by the Institutional Review Board at Wake Forest University and all subjects provided written informed consent.

**SNP Genotyping** Twenty-four SNPs were genotyped in 1168 African American cases (851 with diabetic nephropathy and 317 with type 2 diabetes lacking nephropathy) and 871 non-diabetic controls. SNPs were chosen based on their ability to capture genetic information for African (Yoruban) and European (CEU) populations in Hapmap (www.hapmap.org) using Tagger (Haploview(16)). Tagged SNPs had a minor allele frequency greater than 0.05 and captured an inter-SNP $r^2$ value > 0.8 for known polymorphisms in the region. Three SNPs, rs17300539, rs4632532, rs266729, were included based upon previous association with type 1 diabetic nephropathy or insulin resistance syndrome phenotypes(13; 17). Thirty-four SNPs were initially chosen for analysis. Two SNPs failed design, 6 SNPs could not be incorporated into a multiplex and 2 SNPs were eliminated due to low genotyping efficiency. The genotyped SNPs captured at least 64% of the variation in the Yoruban HAPMAP sample. SNPs were genotyped using the MassARRAY genotyping system (Sequenom Inc., San Diego, CA). PCR primers were designed using the MassARRAY Assay Design 3.4 Software (Sequenom Inc., San Diego, CA). All cases and controls were genotyped at 70 admixture informative markers (AIMs) to estimate the percentage of African ancestry for each individual.(18; 19).

**DNA Sequencing** The associated SNP, rs182052, was sequenced in 175 African Americans with diabetic nephropathy to verify the accuracy of the genotype calls. The region was PCR amplified, products were purified, and directly sequenced using Big Dye Ready Reaction Mix on an ABI3730xl sequencer (Applied Biosystems, Foster City, CA). Sequence data was visualized using Sequencher Software version 4.6 (GeneCodes Corporation, Ann Arbor, MI).

**Statistical Analysis** Biometric data were compared using a Kruskal-Wallis one-way ANOVA on Ranks (SigmaStat, Systat Software, Inc, San Jose, CA) with a Dunn’s Method multiple comparison test. Age at onset of type 2 diabetes in the diabetic nephropathy and type 2 diabetes cases was compared using a Mann-Whitney Rank Sum Test (SigmaStat). Each SNP was tested for departures from Hardy-Weinberg Equilibrium (HWE) using the chi-square goodness of fit test in the statistical analysis program SNPGWA (www.phs.wfubmc.edu)(20). Tests for genotypic association were performed on each SNP individually using SNPGWA-ADIMIX, a component of the SNPGWA program which includes the capability to perform association calculations adjusting for covariates. Genotypic association reported here is for analyses incorporating adjustment for ancestry proportions. The primary inference is based on the 2 degree of freedom global test of genotypic association. If significant, then the individual genetic models (dominant, additive and recessive) were examined for context. This is consistent with the Fisher's protected least significant difference (LSD) multiple comparisons procedure.
Percentage of African ancestry was computed from 70 AIMS using the program Frappe(18; 19). The influence of other possible covariates: age, BMI and gender, on evidence of association was tested using SNPGWA-ADMIX. Two SNP haplotype analysis was completed using the program Dandelion (www.phs.wfubmc.edu). Linkage disequilibrium was calculated as defined by Gabriel (21) with the program Haploview(16).

We computed a series of Cox proportional hazards models and the corresponding likelihood ratio statistics to test for associations between adiponectin polymorphisms and age at type 2 diabetes onset for 1) diabetic nephropathy cases, 2) type 2 diabetes (no nephropathy) cases, and 3) type 2 diabetes and diabetic nephropathy cases combined. Here, age at type 2 diabetes onset was computed for the above three conditions and was contrasted with the age of non-diabetic controls at time of enrollment; specifically, controls had “age at onset of type 2 diabetes” censored at age of enrollment. The hazards ratio (HR) and corresponding 95% confidence interval (CI) was computed as $\hat{\exp} \left[ \hat{\beta} \right]$, and $\exp \left[ \hat{\beta} \pm 1.96 \times SE \left( \hat{\beta} \right) \right]$, respectively. Tests for association and the estimates for the hazards ratio were computed without covariate adjustment and adjusting for gender, BMI and admixture estimates. As discussed above, the genetic models are defined relative to the minor allele frequency.

RESULTS

Descriptive data for participants is summarized in Table 1. Diabetic nephropathy and type 2 diabetes cases were older and had more females compared to the non-diabetic controls. Type 2 diabetes cases had increased BMI compared to diabetic nephropathy cases and non-diabetic controls. The average age at type 2 diabetes onset for cases with diabetic nephropathy was less than that of cases with type 2 diabetes without nephropathy.

Twenty-four SNPs in the adiponectin gene were successfully genotyped in diabetic nephropathy cases and non-diabetic controls. None of the SNPs departed from HWE after correction for multiple comparisons. Two SNPs, rs182052 and rs3821799, showed evidence of association with diabetic nephropathy (Supplemental Table 1). SNP rs182052 was associated with diabetic nephropathy in the 2 degree of freedom test ($P=0.002$) and under the dominant model ($P=0.002$, odds ratio (OR)=$1.37$; 95% confidence interval (CI)=$1.13 – 1.67$) (Table 2). SNP genotype calls for rs182052 were verified by direct DNA sequence analysis in 175 cases with diabetic nephropathy to ensure that the slight departure from HWE in the cases was not the result of erroneous genotyping. Genotypes observed from DNA sequencing were 100% concordant with genotypes generated from the Sequenom MassArray system. The SNP rs3821799 was also associated ($P=0.039$) but did not retain statistical significance after adjusting for multiple comparisons.

SNP rs182052 was associated with BMI and waist measures in Hispanic Americans in the Insulin Resistance and Atherosclerosis (IRAS) Family Study (22). To ascertain whether the association of rs182052 with diabetic nephropathy reflected an association with BMI or other phenotypes known to be associated with increased risk of type 2 diabetes or diabetic nephropathy, genotypic association was evaluated with individual
adjustments for BMI, gender, and age at exam (Table 2) and showed little effect on the evidence of association. We also tested association after simultaneously adjusting for admixture, BMI, gender and age. Evidence of association between rs182052 and diabetic nephropathy remained significant, (P =0.0019, OR=1.46, CI=1.15-1.86) under the dominant model.

To discern whether rs182052 was associated with nephropathy or diabetes in our African American sample, this SNP was tested for association in 317 African Americans with longstanding type 2 diabetes without nephropathy versus the 871 non-diabetic controls. This SNP was associated with type 2 diabetes under the dominant model (P= 0.0054; OR=1.46, CI=1.12-1.91) after adjustment for African ancestry proportions and the association remained significant with adjustment for all four covariates (P=0.0056, OR=1.54, CI=1.13-2.09) (Supplemental Table 2). When evaluating all 1,168 African American diabetic individuals (851 with diabetic nephropathy and 317 with type 2 diabetes without nephropathy), rs182052 remained associated with type 2 diabetes under the dominant model (P= 0.0003, OR=1.40, CI=1.17-1.67) with adjustment for proportion of African ancestry (Table 2). Individual and combined adjustment for African ancestry, age, BMI, and gender did not significantly alter this result. In order to clarify whether the association was with type 2 diabetes only or also with nephropathy, we tested rs182052 for association between the two groups of case individuals, diabetic nephropathy and type 2 diabetes lacking nephropathy. There was no association detected (P=0.87, OR= 0.98; CI=0.73-1.30), indicating that the rs182052 is associated with type 2 diabetes status, and not nephropathy.

The linkage disequilibrium (LD) structure for the adiponectin gene in African American non-diabetic controls is shown in Supplemental Figure 1. African Americans have one large block at the 3' end of the gene and 4 smaller blocks surrounding it. The associated SNP, rs182052, is located in a small LD block containing one other known SNP, rs266729, and covering the promoter.

A Cox proportional hazards model was used to determine the relative risk for early age at onset of type 2 diabetes for genotypes at the associated SNP, rs182052 (Table 3). Significant risk for earlier onset of type 2 diabetes was detected under the dominant model (P=0.0031-0.0002; relative risk (RR)=1.26-1.41) for the diabetic nephropathy and type 2 diabetes case groups individually, and when the two groups were combined (diabetic nephropathy + type 2 diabetes). Significant risk was also found with age at onset of ESRD under the dominant model, (P=0.0113; RR=1.20). Relative risk increased when the data were adjusted for gender, BMI and admixture (P=0.0084; RR=1.21). Pearson’s correlation coefficient, (r = 0.56; P<0.001) indicated a positive correlation between the age at onset of type 2 diabetes and the age at onset of ESRD, suggesting that early age of onset of ESRD may be dependent upon an early age of onset of type 2 diabetes. We confirmed this by calculating the relative risk of age at onset of ESRD after adjustment for age at onset of T2DM and saw no significant risk (Table 3). When the survival distribution function is plotted versus the age at onset of type 2 diabetes (Figure 1), individuals with the minor allele A (A/A or A/G genotypes) have an earlier age at onset of type 2 diabetes, compared to
individuals who are homozygous for the G allele (G/G genotype).

DISCUSSION
We have evaluated association between adiponectin gene polymorphisms and type 2 diabetes and diabetic nephropathy in a large sample of African Americans. We observed evidence of association between the rs182052 polymorphism in intron 1 in African Americans with type 2 diabetes. This evidence of association remained significant after adjustment for African ancestry, age, BMI and gender. In the fully adjusted models, the P-value for association of rs182052 with type 2 diabetes was 0.0005, a level of significance that survives even stringent Bonferroni adjustment. Survival analysis revealed association between the minor allele and earlier age at onset of type 2 diabetes. Previous studies of association between adiponectin polymorphisms and type 2 diabetes or diabetic nephropathy have evaluated European-derived or Asian populations, with only one study in non-European South Africans (14) and one study with a small number of African Americans (15). To our knowledge, this is the first study that has addressed adiponectin gene polymorphism associations with type 2 diabetes and diabetic nephropathy in African Americans.

In our case samples, allele frequencies of rs182052 deviated slightly from Hardy-Weinberg Equilibrium. This may be the result of differing allele frequencies in the European and African ancestral populations. In HapMap (www.hapmap.org), the Yoruban population has a minor allele frequency of 0.395 at this SNP, whereas the European population has a minor allele frequency of 0.353. This difference underscores the importance of including adjustment for African Ancestry in analyses of African American populations. In our cases and controls, the percentage of African ancestry was similar between the two groups of cases and the controls (80 ± 10 – 82 ± 10). This study was well powered to detect associations consistent with complex genetic traits with over 1168 (combined) type 2 diabetes and diabetic nephropathy cases and more than 871 non-diabetic controls. With this large sample size, we had >80% power to detect an odds ratio of 1.3 to 1.5 under the dominant model of association within a range of minor allele frequencies (0.1-0.3). While a number of the genotyped SNPs had low minor allele frequencies (<0.05) and would contribute to a reduction in power, the associated SNP was relatively common (minor allele frequency = 0.348 in controls) and the low P-value contributes a high level of confidence in this association.

Previous studies have reported association between adiponectin polymorphisms and type 2 diabetes (14; 15; 23-26) and diabetic nephropathy(13). These studies have primarily detected association with SNPs in the promoter region, (rs17300539 and rs266729) or in exons, (rs22517766 and rs1501299). With our African American sample we have the ability to detect association with both type 2 diabetes and diabetic nephropathy. We tested three of these SNPs, rs17300539, rs266729, and rs1501299 and found no significant evidence for association. That the association in our African American collection is at a different SNP is not unexpected. This may reflect ethnic differences in adiponectin gene structure as discussed by Ukkola et al.(15) in their evaluation of African Americans from the HERITAGE study. The lack of two distinct
LD blocks in the adiponectin gene in our African American sample, compared to that previously demonstrated in European-derived samples (27), also supports this conclusion. In addition, Woo et al. (28) identified 9 SNPs in the adiponectin gene that had significantly different minor allele frequencies (P<0.001) between African Americans and European Americans. These genetic data are further supported by evidence that African Americans have reduced plasma adiponectin concentrations compared to other ethnic groups (Europeans, Japanese and Pima Indians) (29). The potential for ethnic differences in the adiponectin gene emphasizes the need to study genetic associations in multiple populations, particularly African Americans.

While association with type 2 diabetes has not been previously reported with rs182052, this SNP has been associated with BMI and waist measures in Hispanic Americans in the IRAS Family Study (22). To ascertain whether the association we observed was the result of type 2 diabetes and not increased BMI, we adjusted for BMI in the association analysis. The evidence of association remained significant after BMI adjustment, indicating that the difference in adiposity between cases and controls is not driving the association and that the mechanism is via some other pathway. In addition, rs182052 is in the same haplotype block as rs266729 (Supplementary Figure 1) in African Americans, a SNP with prior reports of association with type 2 diabetes (11). Haplotype analysis of these two SNPs revealed no association with type 2 diabetes or diabetic nephropathy (Supplementary Table 3).

Woo et al. (28) and Heid et al. (27) have demonstrated association between the minor allele of rs182052 (A) and reduced levels of plasma adiponectin in European-derived American adolescents and healthy Europeans, respectively. In our study, individuals with the minor allele (A/A or A/G, genotypes) had earlier onset of type 2 diabetes. If the minor allele is contributing to type 2 diabetes in our samples by reduced levels of plasma adiponectin, this would be consistent with previous studies that have demonstrated an association between hypo-adiponectinemia and increased risk of type 2 diabetes (24; 30). We do not have serum adiponectin concentrations for the subjects in our study, however it would be interesting to determine whether rs182052 contributes to plasma adiponectin concentrations in our collection of African Americans. It should be noted that Woo et al. (28) detected no association between rs182052 and plasma adiponectin levels in a comparably sized sample of African American adolescents.

A primary issue with the observations we have made is whether the observed association reflects an association between rs182052 and type 2 diabetes, diabetic nephropathy, or both. We have sampled a relatively large number of African American subjects with diabetic nephropathy and a smaller collection with type 2 diabetes and no nephropathy to attempt to ascertain whether this gene is associated with nephropathy or diabetes. As the SNP was associated in subjects with diabetic nephropathy and subjects with type 2 diabetes lacking nephropathy, we believe that the data are most consistent with the rs182052 variant contributing to type 2 diabetes susceptibility in African Americans. This is supported by the Cox model’s relative risk, which indicated increased risk for earlier age at onset of type 2 diabetes.
Additionally, we compared allele frequencies of rs182052 between the diabetic nephropathy cases (n=851) and the type 2 diabetic (no nephropathy) cases (n=317) and observed no evidence of association. Although this analysis has less power than the initial case-control analysis, it does support our conclusion that rs182052 is associated with type 2 diabetes in African Americans.

We observed association between a variant in the adiponectin gene and type 2 diabetes in African Americans. This SNP, rs182052, has not previously been associated with diabetes or diabetic nephropathy in other populations, although it has been associated with plasma adiponectin levels. That other variants previously associated with diabetes or diabetic nephropathy in Europeans were not associated in our African American population emphasizes the potential that there are likely ethnic differences in the LD structure of the adiponectin gene.

ACKNOWLEDGEMENTS
This study was supported in part by the General Clinical Research Center of the Wake Forest University School of Medicine grant M01 RR07122, R01 DK066358 (DWB), R01 DK053591 (DWB), and R01 DK 070941 (BIF). MAB was supported by F32 DK080617 from the NIDDK.
Table 1: Demographic characteristics of African Americans cohort. * mean is significantly different from non-diabetics \( (p < 0.05) \). † mean is significantly different \( (p < 0.05) \) from the T2DN cases. AO T2DM = Age at onset of Type 2 DM; AO ESRD = Age at onset of ESRD

<table>
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<tr>
<th>Subjects</th>
<th>(N)</th>
<th>Age</th>
<th>BMI</th>
<th>% Female</th>
<th>AO T2DM</th>
<th>AO ESRD</th>
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<tr>
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<td>29.5 ± 7.0</td>
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<td></td>
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<tr>
<td>Diabetic Nephropathy (DN)</td>
<td>851</td>
<td>61.8 ± 10.0*</td>
<td>29.4 ± 6.8</td>
<td>62.7%</td>
<td>41.0 ± 12.0</td>
<td>58.5 ± 10.2</td>
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<tr>
<td>T2DM (no Nephropathy)</td>
<td>317</td>
<td>58.6 ± 11.5*</td>
<td>32.9 ± 7.1*</td>
<td>65.5%</td>
<td>43.8 ± 12.0†</td>
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</table>
Table 2: Genotypic association with diabetic nephropathy and type 2 diabetes. Genotypic association for rs182052 after individual adjustment for admixture, age, BMI, and gender in 851 T2DN cases versus 871 non-diabetic controls and T2DN cases plus an additional 317 T2DM (no nephropathy) (total cases = 1168) versus the same 871 non-diabetic controls. Genotypic association is also shown for all four covariates simultaneously (combined). OR = odds ratio, CI = 95% confidence interval.

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<th>Recessive</th>
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<td>OR</td>
<td>CI</td>
<td>P-value</td>
<td>OR</td>
<td>CI</td>
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<td>0.060</td>
<td>0.0019</td>
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<td>1.13-1.67</td>
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<td>0.0009</td>
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<td>1.18-1.89</td>
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<td>1.26</td>
<td>1.06-1.49</td>
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<td>0.0004</td>
<td>1.46</td>
<td>1.19-1.80</td>
<td>0.0110</td>
<td>1.22</td>
<td>1.05-1.42</td>
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<tr>
<td></td>
<td>Gender</td>
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<td>0.0013</td>
<td>1.38</td>
<td>1.13-1.67</td>
<td>0.0378</td>
<td>1.16</td>
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<td></td>
<td>Combined</td>
<td></td>
<td></td>
<td>0.0019</td>
<td>1.46</td>
<td>1.15-1.86</td>
<td>0.0114</td>
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<td>T2DM + DN vs non-diabetic</td>
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<td>0.061</td>
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<td>1.17-1.67</td>
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<tr>
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<td>0.0002</td>
<td>1.50</td>
<td>1.21-1.86</td>
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<td>0.0002</td>
<td>1.41</td>
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<td>1.03-1.34</td>
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<td>1.19-1.84</td>
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Table 3: Survival Analysis to estimate risk of early age at onset of T2DM and ESRD. P-values and relative risk (RR) are reported for the dominant, additive and recessive models both prior to and following adjustment for gender, BMI and admixture proportions. Analysis of age at onset of ESRD is also adjusted for age at onset of T2DM.

<table>
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<tr>
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<th>Recessive</th>
<th>Additive</th>
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<tr>
<td></td>
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<td>RR</td>
<td>P-value</td>
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<tr>
<td><strong>Age at Onset of T2DM</strong></td>
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<tr>
<td>DN</td>
<td>0.0003</td>
<td>1.29</td>
<td>1.0000</td>
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<tr>
<td>T2DM</td>
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<td>1.41</td>
<td>0.9714</td>
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<td>DN+T2DM</td>
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<td><strong>After Adjustment</strong></td>
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<tr>
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<td>DN+T2DM</td>
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<td><strong>Age at Onset of ESRD</strong></td>
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<tr>
<td>DN</td>
<td>0.0113</td>
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<td><strong>After Adjustment for gender, BMI and admixture</strong></td>
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<tr>
<td>DN</td>
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<td>0.1148</td>
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<tr>
<td>DN</td>
<td>0.86</td>
<td>0.99</td>
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**Figure 1:** Kaplan-Meier plots for age of onset of type 2 diabetes by genotype for (A) diabetic nephropathy cases (n=851) and controls (n=871), (B) type 2 diabetes cases (n=317) and controls, and (C) diabetic nephropathy + type 2 diabetes cases (n=1168) and controls.
REFERENCES


