

ARIC Manuscript Proposal # 984

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1.a. Full Title: Genetic Risk Factors for Nephropathy in the ARIC Study: GLUT1, ZO-1 and NPHS2

b. Abbreviated Title (Length 26 characters): Study of Microalbuminuria Candidate Genes

2. Writing Group (list individual with lead responsibility first):

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3. Timeline:

Determination of microalbuminuria from all visit 4 urine samples (N=10,902) is being performed by the laboratory of Michael Steffes (Univ. of Minnesota) and is scheduled to be completed April 2004. The first process batch (N=6,288 individuals) consisting of all of Jackson, MS and Washington Co., MD and diabetics from Forsyth Co., NC and Minneapolis, MN is expected to be completed by December 31, 2003. Genotyping of single nucleotide polymorphisms (SNPs) of *Glucose Transporter-1 (GLUT1)*, *Zonula Occludens-1 (ZO1)*, and *Podocin (NPHS2)* for diabetics, African-Americans, and a subset of Caucasians in the CHD case-control studies (N=8948) is being performed under the supervision of Drs. Linda Kao (Johns Hopkins) and Alan Shuldiner (Univ. of Maryland) with December 2003 the expected date of completion for *GLUT1* and February 2004 the expected date of completion for *ZO1* and *NPHS2*. The remaining data for these analyses are already available as part of ARIC. We project that the analyses and writing will take place over the six months following completion of genotyping and urinalysis.

4. Rationale:

Renal disease is a growing epidemic in the United States. The incidence and prevalence of ESRD have doubled in the past 10 years and are expected to increase steadily(1). Diabetic nephropathy (DN) is the single most common type of renal disease in new dialysis patients and accounts for more than 1/3 of all patients with ESRD(2-4). The proportion of incident dialysis patients with DN as their primary diagnosis is also increasing dramatically, from 33% in 1990 to 43% in 1998(2). Hypertension is the second most common cause of renal failure and was the primary diagnosis in 26.4% of incident dialysis patients from 1995-99(5). A population

particularly affected by kidney disease due to diabetes and hypertension is the U.S. African-American population, which has 3 times the incidence rate of U.S. whites(3).

Microalbuminuria (MA) is the earliest clinical manifestation of DN and refers to an increase in albumin excretion above 30 mg/day and below 300mg/day(4). In an untimed urine sample, an albumin-to-creatinine ratio (ACR) can be calculated that closely approximates albumin excretion in a 24-hour urine sample (6;7). Individuals with MA have been shown to have increased risk of cardiovascular events (8-11) regardless of diabetic status (12) and to be at increased risk for renal disease progression in those with type 1 diabetes (13;14) and type 2 diabetes(15). Additionally, there is evidence that even high-normal albuminuria may be associated with an adverse cardiovascular risk profile (16). It has been estimated that the prevalence of MA in the U.S. population is 7.8%; in diabetics it is 28.8%, and in hypertensives it is 16.0%(17). MA was associated with older age, African-American and Mexican-American ethnicity, presence of diabetes, hypertension, and elevated serum creatinine concentration(17). MA is of particular public health and clinical significance and necessitates more effort to understand its etiology and consequences, especially among high-risk populations.

There is strong evidence that inherited factors contribute to DN and susceptibility for other kidney diseases. Familial aggregation of nephropathy has been demonstrated in all major etiologies of end-stage renal disease (ESRD) and in every ethnic group evaluated(18). Epidemiological and family studies indicate genetic factors play an important role in the development of diabetic nephropathy (19-21). There is especially compelling evidence in African-Americans for a genetic contribution to renal disease; evidence demonstrates that ESRD clusters independently from the systemic diseases of hypertension, diabetes mellitus, HIV infection, and systemic lupus erythematosus, furthermore disparate etiologies of ESRD exist within families from widely separated geographic regions of the US. Based on recent genetic findings, we are interested in examining genetic variation of *GLUT1*, *ZO1*, and *NPHS2* for possible associations with nephropathy in ARIC.

GLUT1 is located on chromosome 1p34.2 and has been identified as the major glucose transporter in renal mesangial cells, (22;23) and the association of increased *GLUT1* expression with renal hypertrophy and increased extracellular matrix (ECM) formation by rat renal mesangial cells mimics the human DN phenotype(24). *GLUT1* has widespread tissue distribution with highest levels in erythrocytes and vascular endothelium(25). There is currently no data on *GLUT1* protein levels and either DN or *GLUT1* variation. Clinical studies have focused on DN and the *GLUT1* intronic *XbaI* (-) polymorphism, a G-to-T transversion which occurs in intron 2 and corresponds to dbSNP# rs841853. There has been strong evidence that the *XbaI* (-) allele of the *GLUT1* gene is a marker of susceptibility of DN with NIDDM in a Chinese population (26) and IDDM in Caucasians in a UK population (27) and a US population(28). Yet, there have also been conflicting results in disparate populations in Denmark(29) and Poland(30). Allele frequencies of the *XbaI* (-) polymorphism have been around 30-35% (26;28-30) except for the British population which had a frequency of 53% population(27). One limitation has been that analysis of a single intronic polymorphism is not sufficient to capture the full variation in the candidate gene.

Recently, Ng et al. examined in a case-control study 492 type 1 diabetic patients possible associations of DN and 6 *GLUT1* SNPs in a case-control study(28). The authors identified three putative *GLUT1* enhancers with sequence similarity to mouse homologs. In addition to *XbaI*, the

authors examined an enhancer-1 SNP, an enhancer-3 SNP, an exon 2 (HaeIII) SNP, and SNPs 1 and 2 of enhancer-2 located in intron 2 in close proximity to the XbaI marker. The authors found an increased risk of diabetic nephropathy among XbaI (-) homozygotes (OR 1.83; 95% CI: 1.01-3.33) and also among those with enhancer-2 SNP1 AA genotypes (OR 2.38; 95% CI: 1.16-4.90). There was strong linkage disequilibrium found between enhancer-2 SNP1 and the XbaI SNP; those homozygous for risk alleles at both SNPs were at increased risk of DN (OR 2.40; 95% CI: 1.13-5.07). The Ng et al. study strengthened the evidence for an association between *GLUT1* and DN though only rudimentary haplotype analysis was performed involving the XbaI marker and the enhancer-2 SNP 1 marker, with non-significant results. We propose to examine SNPs in the above mentioned enhancers with additional exonic and intronic SNPs to provide more sufficient coverage of *GLUT1* in our sample. We are particularly interested in XbaI and the enhancer-2 SNP 1. Additional SNPs were chosen based on previous publications, allele frequency, and/or SNP location/possible functionality (e.g. enhancer / promoter region). The increased SNPs and coverage of *GLUT1* will allow us to perform more precise haplotype frequency analysis.

Additionally, we are interested in examining polymorphisms of *Zona occludens 1*, a tight junction protein located on 15q13.1. ZO-1 is a 200 kDa protein located on the cytoplasmic membrane surfaces of vertebrate intercellular tight junctions; though little is known of its function, a multi-domain signaling protein homologous to a *Drosophila* tumor suppressor gene and several other mammalian membrane-associated proteins has been predicted from its cDNA sequence(31). Fluorescence in situ hybridization studies in patients with either the Prader-Willi syndrome or the Angelman syndrome demonstrated that *ZO-1* is located in close proximity to the causative chromosomal region associated with these syndromes(32). The distribution of ZO-1 is known to be altered in podocytes in proteinuric states. Additionally, ZO-1 may play a role with transforming growth factor-beta1 in congenital kidney malformations (33). As microalbuminuria is evidence of pathologic changes of filtration of protein across glomerular membranes and intercellular junctions, polymorphisms of *Zonula occludens 1* are plausible candidates for playing a role in DN. Recently, Bhandari et al. presented results at the 2002 meeting of the American Society of Nephrology (34) and the 2002 meeting of the American Diabetes Association (35) demonstrating an association between chromosomal region 15q and microalbuminuria in 335 diabetic and non-diabetic Mexican-American study participants from the San Antonio Family Diabetes Study. Their results suggest *ZOI* as a probable candidate gene associated with microalbuminuria. We propose to examine several *ZOI* exonic and intronic SNPs and possible associations with microalbuminuria in diabetics and African-Americans in ARIC.

Furthermore, we are also interested in the *Podocin* gene (*NPHS2*) located on chromosome 1q25-q31. *NPHS2* mutations have been found to be associated with autosomal recessive steroid-resistant nephrotic syndrome(36) and FSGS (including sporadic FSGS)(37). Transmission appears to be recessive for the majority of mutations(38). The gene product is an integral membrane protein that may play a essential role in maintaining the structural integrity of the fenestrations and slit diaphragms of the renal glomeruli for proper filtration. There are few demonstrated functional polymorphisms in these SNPs. The most common SNP is the R229Q polymorphism, which causes a recessive form of nephrotic syndrome, with possible late-onset(39). The R229Q variant may have an allele frequency in control populations of 3.6% (39). However, there has been recent evidence that some carriers of the R229Q mutation have

evidenced of albuminuria(40). We hypothesize that R229Q carriers, exposed to the right environmental insults (e.g. diabetes, hypertension, etc) may develop proteinuria.

ARIC provides an excellent opportunity to study genetic risk factors for nephropathy using the visit 4 stored urines to determine albuminuric status. Albumin-to-creatinine ratios (ACR) will be determined for 10,902 individuals from visit 4 stored urine specimens. Approximately 2000 diabetics and 2200 African-Americans are included in this sample. We will perform a case-control study utilizing our sample. Methods for measuring ACR have been described previously and will be performed by Mike Steffes at the University of Minnesota (9). The primary outcome will be defined as micro- or macroalbuminuria. Sex-specific cutpoints will be chosen to delineate normoalbuminuria from microalbuminuria: 17 ug/mg for men and 25 ug/mg for women. The upper boundary delineating microalbuminuria from overt proteinuria will be 250 ug/mg for men and 355 ug/mg for women(41). The sex specific cutpoints have been shown to correspond well with AER-based definitions of microalbuminuria(41). Serum creatinine, lipid profile (total cholesterol, LDL, HDL), duration of diabetes, serum glucose, insulin levels, history of cardiovascular disease, blood pressure, and cigarette use will be examined as covariates in the multivariate analysis. We will examine case definitions of 1) micro- and macroalbuminuria and also 2) only macroalbuminuria in order to increase specificity.

The frequency of each marker allele for *GLUT1*, *ZOI*, and *NPHS2* will be compared between those with and without microalbuminuria using a chi-squared test. A two-sample t-test will be used to test for differences in continuous demographic characteristics between patients and control subjects, and a chi-squared test will be used to test for differences in categorical demographic characteristics between patients and control subjects. Simple logistic regression will first be done between microalbuminuria and individual genotypes. Multivariate logistic regression will be used to test for significant associations between microalbuminuria and individual genotypes adjusting for exposure covariates. Gene-gene and gene-environment interactions will also be explored. Additionally, 1) microalbuminuria with visit 4 GFR <60 will be examined as a separate outcome to further increase specificity. Additionally, *GLUT1* genetic variation and 1) serum glucose and also 2) indices of insulin resistance will be examined to determine possible functional polymorphisms associated with glucose control. All analyses will also be done stratified on race, diabetes, gender, and hypertension to avoid overlooking potential interactions.

ARIC also provides an excellent opportunity to study genetic risk factors for the progression of chronic kidney disease (CKD progression) using the visit 1, 2, and 4 plasma creatinine measures and surveillance data for kidney disease hospitalization. As a secondary portion of the proposed study, we will also examine *GLUT1*, *ZOI*, and *NPHS2* genetic variation and their association with decline in renal function. A prospective analysis of genetic risk factors for a rise in plasma creatinine and/or hospitalization for kidney disease during the 9 years of follow-up will be done. The outcome of CKD progression will be a combined incidence of a rise of at least 0.4 mg/dL in plasma creatinine above baseline (after accounting for laboratory differences between visit 4 and prior visits) or a hospitalization for kidney disease. This definition has worked well in prior ARIC analyses(42;43). In addition, we will conduct analyses of the cross-sectional association of genotype with estimated GFR at the baseline visit. GFR will be estimated using the abbreviated Modification of Diet in Renal Disease (MDRD) equation (1;44). Furthermore, to better understand the pathophysiology of nephropathy, we will also examine if genetic associations with albuminuria are indicative of solely renal manifestations of disease or of more

systemic atherosclerosis and subclinical cardiovascular disease by examining associations with carotid intima-media thickness. Sensitivity analyses will also explore a $\geq 30\%$ drop in estimated GFR as an alternative outcome. These parallel analytic approaches will ensure that results are robust to the specific outcome definition. The study population will include approximately 9000 individuals, of whom 3500 are African American and 2000 are diabetic. Within ARIC, it has been shown that early renal function decline is 3 times more likely to develop in blacks than whites (43).

For genetic variation of *GLUT1*, *ZOI*, and *NPHS2*, haplotype frequency analyses will also be performed. Each allele (SNP) is associated with a particular evolutionary history and will have a unique chromosomal background, or haplotype. Haplotype-based analyses provide increased information over consideration of single markers. Haplotype frequencies for the marker combinations will be estimated for cases and controls separately via an Expectation-Maximization algorithm(45). Chi-squared values and permutation test significance levels for individual haplotype frequency comparisons between cases and control groups will be ascertained. An omnibus likelihood ratio test, which examines the differences in haplotype frequency profiles between the case and control groups, will be pursued(45).

The ability to examine gene-gene and gene-environment interactions will make this study especially interesting. The potential to examine pathways of genes and nephropathy in a population-based sample of diabetics and those that have been underrepresented in studies thus far will make this analysis particularly pertinent. Furthermore, by examining both albuminuria, CKD progression, and atherosclerosis, we can better understand the possible role *GLUT1*, *ZOI*, and *NPHS2* may play in nephropathy. If some of these variants predict proteinuria, it will be important to see if they also predict subsequent atherosclerosis. This will help shed light on whether albuminuria is only a marker or a causal factor in atherosclerosis. Potential confounding by age, race, gender, socioeconomic factors, blood pressure, hypertension, diabetes, serum glucose, BMI and lipids will be controlled for. All analyses will be done stratified on race, diabetes, gender, and hypertension to avoid overlooking potential interactions.

5. Main Hypothesis/Study Questions:

To examine the role of genetic variation of *GLUT1*, *ZOI*, and *NPHS2* and their association with nephropathy in ARIC. Specifically:

- 1) a case-control study of prevalent of micro- and macroalbuminuria
- 2) a prospective study of CKD progression (kidney disease hospitalization / elevation of serum creatinine)
- 3) association with baseline and progression of carotid atherosclerosis will be tested for variants which are associated with proteinuria. This will help shed light on whether albuminuria is only a marker or a causal factor in atherosclerosis.

6. Data (variables, time window, source, inclusions/exclusions):

Data analysis will be performed by C. Hsu at the Johns Hopkins School of Hygiene & Public Health.

Variables needed (available at JHU): plasma creatinine and time of collection, center, age, gender, race, blood pressure, physical activity, medication use, alcohol and cigarette use, lipid profiles, blood glucose, insulin, anthropometric data, lipid profiles, carotid intima-media thickness, medical history data (diabetes and cardiovascular events) and hospitalization for cardiovascular disease, genotypes for *GLUT1*, genotypes for *ZOI*, genotypes for *NPHS2*, and ACRs from visit 4 urines.

7.a. Will the data be used for non-CVD analysis in this manuscript? ☐ Yes ☒ No

b. If Yes, is the author aware that the file ICTDER02 must be used to exclude persons with a value RES_OTH = “CVD Research” for non-DNA analysis, and for DNA analysis RES_DNA = “CVD Research” would be used? ☒ Yes ☐ No
(This file ICTDER02 has been distributed to ARIC PIs, and contains the responses to consent updates related to stored sample use for research.)

8.a. Will the DNA data be used in this manuscript? ☒ Yes ☐ No

(Note: DNA from the Brancati ancillary study (N=8,948) will be used for this study. This will include all Diabetics and African-Americans.)

8.b. If yes, is the author aware that either DNA data distributed by the Coordinating Center must be used, or the file ICTDER02 must be used to exclude those with value RES_DNA = “No use/storage DNA”? ☒ Yes ☐ No

9. The lead author of this manuscript proposal has reviewed the list of existing ARIC Study manuscript proposals and has found no overlap between this proposal and previously approved manuscript proposals either published or still in active status. ARIC Investigators have access to the publications lists under the Study Members Area of the web site at: <http://bios.unc.edu/units/csc/ARIC/stdy/studymem.html>

☒ Yes ☐ No

10. What are the most related manuscript proposals in ARIC (authors are encouraged to contact lead authors of these proposals for comments on the new proposal or collaboration)?

MS#223 Risk factors for decreased renal function in the ARIC Study

MS#868 Lipid-related genetic risk factors for decline in renal function in African-Americans in the ARIC Study

11. Manuscript preparation is expected to be completed in one to three years. If a manuscript is not submitted for ARIC review at the end of the 3-years from the date of the approval, the manuscript proposal will expire.

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