ARIC Manuscript Proposal # 1065

PC Reviewed: _02/11/05	**Status: __A__**	**Priority: __2__**
SC Reviewed: __02/14/05	**Status: __A__**	**Priority: __2__**

1.a. Full Title: Variations of LDLR gene expression and ApolipoproteinE isoforms as cardiovascular risk

b. Abbreviated Title (Length 26 characters): LDLRgenotype, ApoE isoforms and cardiovascular risk

2. Writing Group: Hind Muallem, Kari North, David Couper, Gerardo Heiss, Eric Boerwinkle (others are welcome)

Lead: **: Dr. Nobuyo Maeda**
Department of Pathology and Laboratory Medicine
UNC- Chapel Hill
701 Brinkhous-Bullitt Bldg
CB#7525
Chapel Hill, NC27599-7525
Phone: 919-966-6912
Fax: 919-966-8800
nobuyo@med.unc.edu

3. Timeline: Anticipate data will be available:
Analysis to be completed:
First draft anticipated:

4. Rationale:

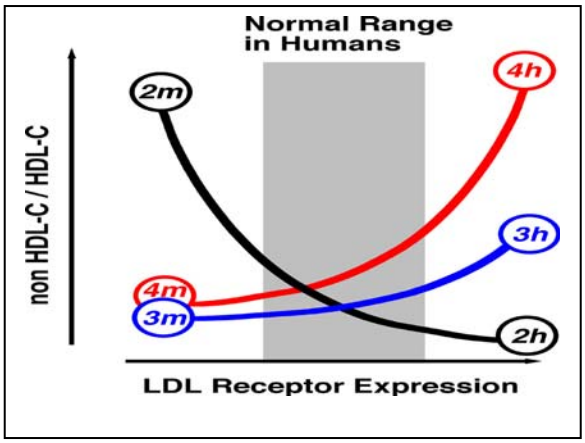
The primary objective of the proposed study is to dissect the interrelationships between the low-density lipoprotein receptor (LDLR) gene polymorphisms and apolipoprotein E (apoE) isoforms in atherosclerosis risk in humans.

ApoE plays a pivotal role in the clearance of lipoprotein particles from the blood circulation (1). In humans, there are three common isoforms, apoE2, apoE3 and apoE4. Due to the difference in amino acids, apoE isoforms have different binding affinity for the LDLR, with apoE4 having the highest binding affinity followed by apoE3. ApoE2 has much reduced receptor-binding affinity compared to the other two isoforms. Despite the low affinity, however, individuals having an apoE2 have a lower plasma cholesterol and risk of atherosclerosis, except for about 5% of apoE2 homozygote who exhibit type III hyperlipoproteinemia and atherosclerosis. In general,

individuals with apoE4 have a slightly increased plasma LDL cholesterol and hence increased risk of atherosclerosis (2-6). The reason for this paradoxical relationship is not clearly understood.

Using a targeted gene replacement approach, we have developed mice expressing human apoE and fed them a Western-type high fat diet. We found that the genetic increase in expression of the LDLR gene results in the accumulation in plasma of cholesterol-rich, apoE-poor remnant particles in mice expressing human apoE4, but not in mice with apoE3. Increased LDLR also caused the mice with human apoE4, but not with apoE3, to develop severe atherosclerosis (7). In contrast, mice expressing apoE2 exhibit type III hypercholesterolemia and atherosclerosis when their LDLR expression is normal. These conditions in the apoE2 mice are, however, ameliorated completely by an increased LDLR expression (8). Thus data in mice suggest that there is a complex interaction between apoE genotype and LDLR expression levels of plasma cholesterol and its vascular consequence (9).

Figure 1 summarizes our conclusion that apoE is a determinant of atherogenic risk (represented by the ratio of nonHDL cholesterol and HDL cholesterol) in a previously unrecognized fashion and dependent on the levels of LDLR expression. When mice express relatively low levels of LDLR (left side of abscissa), mice with apoE2 isoforms have a higher atherosclerosis risk than those with apoE3 or apoE4. In contrast, when they express double the amount of LDLR, mice with apoE4 have an increased risk, while risk in mice with apoE2 is reduced (right hand side). Thus, there appears to be a cross over of the relationships between apoE and LDLR expression.



It is reasonable to assume that there are genetically determined variations governing the basal expression levels of LDLR among humans. While the LDLR expression is within a normal range (as illustrated by the gray box in Figure 1) we hypothesize that apoE-LDLR interactions are present in humans similar to those in mice , and thus influence the atherogenic lipd profile.. We aim, therefore, to test this hypothesis.

It has been recognized that common polymorphisms that influence the expression of genes are important contributors to the genetic susceptibility to complex diseases. The promoter and enhancer elements regulate gene expression at the level of transcription. For example, Charlotte et al found that the SNP C59T in the promoter of the LDLR significantly reduces the promoter activity by 40% in both the presence and absence of sterol, while the SNP C124T increases LDLR expression by 60% (10).

The sequences in the 3'UTR, on the other hand, influence the steady state levels of transcripts by affecting their stability. Functional analysis of the 3'UTR variants in the human LDLR has not been reported. Therefore, we are currently examining the differences in gene expression of three common haplotypes, AACG, GGTA and GGCA at four SNP sites in the 3'UTR; namely G44243A, G44695A, C44857T and A44964G. We cloned DNA fragments containing these 3'UTR sequences individually downstream of a GFP reporter gene and have examined GFP expression using an embryonic stem cell based assay system (11). Our preliminary data indicate

that the GFP expression with the AACG and GGTA haplotypes are 59% and 37% respectively, of that with GGCA haplotype. The frequencies of the respective haplotypes in human populations are approximately 26 %, 17 % and 50 %. We have developed a Taqman based genotyping system for these haplotypes.

Our prediction is that individuals with GGCA haplotype have higher basal levels of the LDLR gene expression, and are at higher risk of developing atherosclerosis relative to those who with AACG and GGTA, if they also carry apoE4. In contrast GGCA individuals who also carry apoE2 are protected. We also predict that individuals with GGTA have lower basal levels of the LDLR gene expression and are more susceptible to type III hypercholesterolemia if they are homozygous for apoE2.

5. Main Hypothesis/Study Questions:

Our main study questions are as follows:

I. Variations of LDLR gene expression and ApoE isoforms are associated with fasting levels of total and lipoprotein cholesterol, and triglycerides.

A – To evaluate the relationship between variation in four SNPs of the LDLR gene in humans and fasting level of total cholesterol, triglyceride, LDL-C, and HDL-C.

B- To evaluate the relationship between variations in three SNPs of the Apo E gene in humans and fasting level of total cholesterol, triglyceride, LDL-C, VLDL-C and HDL-C.

C – To evaluate the joint effect (interaction) of the LDLR SNPs, Apo E SNPs on fasting level of total cholesterol, triglyceride, LDL-C, VLDL-C and HDL-C.

II. Variations of LDLR gene expression and ApoE isoforms are associated with intima-media thickness / carotid artery plaque.

A – To evaluate the relationship between variation in four SNPs of the LDLR gene in humans and (a) reader-trend adjusted far wall IMT and (b) presence of carotid artery lesions and acoustic shadowing.

B- To evaluate the relationship between variations in three SNPs of the Apo E gene in humans and (a) reader-trend adjusted far wall IMT and (b) presence of carotid artery lesions and acoustic shadowing.

C – To evaluate the joint effect (interaction) of the LDLR SNPs and Apo E SNPs on (a) reader-trend adjusted far wall IMT and (b) presence of carotid artery lesions and acoustic shadowing.

III. Variations of LDLR gene expression and ApoE isoforms are associated with risk of incidence coronary heart disease.

A – To evaluate the relationship between variation in four SNPs of the LDLR gene in humans and (a) the risk of incident, fatal or non-fatal myocardial infarction and (b) the risk of fatal or non-fatal CHD.

B- To evaluate the relationship between variations in three SNPs of the Apo E gene in humans and (a) the risk of incident, fatal or non-fatal myocardial infarction and (b) the risk of fatal or non-fatal CHD.

C – To evaluate the joint effect (interaction) of the LDLR SNPs and Apo E SNPs on (a) the risk of incident, fatal or non-fatal myocardial infarction and (b) the risk of fatal or non-fatal CHD.

Analytical Strategy to Address Study Questions.

Analyses for Topic I will be completed first, since it will inform the analytic strategy for topics II and III.

We will first test for gross errors in population stratification and deviations from Hardy Weinberg Equilibrium.

Study Question A - Use multiple analytical strategies to determine the main effect of the LDL-Receptor variants on quantitative variation on fasting level of total cholesterol, triglyceride, LDL-C, VLDL-C and HDL-C.

1. Measured genotype approach: Simple association analysis of SNP genotypes with lipid related measures can be performed using the measured genotype approach (Hopper and Mathews 1982, Boerwinkle *et al.* 1986). Having very high power for the simple case of detecting a functional variant is necessary in this case because the reality is that we may be measuring markers in LD rather than the true functional variants or the functional variant may be defined by an extended haplotype for us to discover. We will carry out such analyses using the computer package, SOLAR (Almasy and Blangero 1998). In this approach, SNP genotypes are modeled as fixed effects or covariates and a likelihood ratio is calculated to test whether SNP genotypes exhibit statistically different means. The effects of additional covariates such as sex and age can be simultaneously estimated.

2. Analysis of variance

Study Question B-- Use multiple analytical strateiges to determine the main effect of the Apo E variants on quantitative variation on fasting level of total cholesterol, triglyceride, LDL-C, VLDL-C and HDL-C.

Use the same methods described above for #A.

Study Question C – Use multiple analytical strategies to determin the multiplicative interaction between the LDLR and APOE genotype variants on fasting level of total cholesterol, triglyceride, LDL-C, VLDL-C and HDL-C.

1. Measured genotype approach: Multiplicative interation of SNP genotypes with lipid related measures can be performed using the measured genotype approach (Hopper and Mathews 1982, Boerwinkle *et al.* 1986). We will carry out such analyses using the computer package, SOLAR (Almasy and Blangero 1998). In this approach, SNP genotypes are modeled as fixed effects or covariates and a likelihood ratio is calculated to test whether SNP genotypes exhibit statistically different means. The effects of additional covariates such as sex and age can be simultaneously estimated.

2. Analysis of variance

II. Variations of LDLR gene expression and ApoE isoforms and intima-media thickness / carotid artery plaque.

These analyses will be cross-sectional, stratified by gender and race-ethinicity, and based on ARIC's baseline. LDLR and APOE genotype variants found to be associated with levels of total cholesterol (or an atherogenic lipid profile) will be examined for their association with reader-trend adjusted average far wall IMT, adjusted for age, center, smoking status, and systolic hypertension * antihypertensive treatment. Equivalent analyses will be performed for the prewsence / absence of carotid artery plaque as the dependent variable. Numbers permitting (based on the LDLR and APOE genotype variants to be tested), the presence of acoustic shadowing (a marker of mineralized plaque) will be introduced as an additional level of the dependent variable.

III. Variations of LDLR gene expression and ApoE isoforms and risk of incidence coronary heart disease.

These analyses will be prospecive, stratified by gender and race-ethinicity, and based on ARIC's Visit 1 as the baseline for follow-up time. LDLR and APOE genotype variants found to be associated with levels of total cholesterol (or an atherogenic lipid profile) will be examined for their association with the risk of incident (validated) myocardiaol infarction (fatal or non-fatal). Once it has been verified that the proportionality assumptions are met, covariates in the proportional hazards model will include age, center, smoking status, and systolic hypertension * antihypertensive treatment. Should numbers be insufficient to test a complex assoiation between LDLR and APOE genotype variants on the risk of myocardial infarction, coronary heart diesase will be chosen as the dependent variable.

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

Covariate data, IMT and carotid plaque will be drawn from the basline examination visit. Time to event information will computed since the Visit 1 baseline.

A. LDLR genotype, ApoE genotype on entire ARIC cohort
B. Lipid profile (Total cholesterol level,VLDL cholesterol, LDL cholesterol, HDL cholesterol, and Triglyceride).
C. Information on covariates e.g, race, sex, age, athropometric measures, use of lipid-lowering medication, chronic illness (cancer, CHD, stroke, diabetes, hypertension, autoimmune disease), smoking, and alcohol consumption.

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

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"? __x__ 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://www.cscc.unc.edu/ARIC/search.php

___x___ 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)? None

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.

References.

1 – Weisgraber, K.H., Apolipoprotein E: structure –function relationships. Adv Protein Chem, 1994.45p. 249-302.

2 – Davignon J, Gregg RE, Sing CF. Apolipoprotein E polymorphism and atherosclerosis. Arteriosclerosis. 1988;8:1-21.

3 – Boerwinkle E, Utermann G. Simultaneous effects of apolipoproteins E polymorphism on apolipoprotein E, apolipoprotein B, and cholesterol metabolism. Am j Hum Genet. 1988;42:104-112.

4 – Weisgraber KH. Apolipoprotein E : structure-function relationship.Adv protein Chem. 1994;45:249-302.

5 – Bohnet K, pilot T, Visvikis S, Sabolovic N, Siest G.Apolipoprotein (apo) E genotype and apo E concentration determine binding of normal very low density lipoproteins to HepG2 cell surface receptors. J Lipid Res.1966;37:1316-1324.

6 – Knouff C, Hinsdale ME, Hezdour H, Altenburg M, Watanabe M., Quafort SH, Sullivan PM, Maeda N. Apo E structure determines VLDL vlearance and atherosclerosis risk in mice. J Vlin Invest. 1999;103:1579-1586.

7.- . Malloy I, Altenburg M, Knouff C, Lanningham-Foster L, Parks S, Maeda N. harmful effect of increased LDLR expression in mice with human Apo E*4 but not Apo E*3. Arterioscler Throm Vasc Biol. January 2004:91-97.

8.-. Knouff C, Sudi,M, Wilder J, Altenburg M, Maeda N. Doubling expression of the low density lipoprotein receptor by truncation of the 3'-untranslated region sequence ameliorates type III hyperlipproteinemia in mice expressing the human Apo E2 isoform. J of Biol chem. February 9: 3856-3862.

9- Altenburg M, Mallo Sudi, Knouff C, Parks J, Maeda N. Apo E4 trapping by the low density lipoprotein receptor.

10 - Charlotte L, Armand V, Christiaan F, Rochelle TJournal , J.N.P. de Villiers, Renate H, Jingwen L, A.David M, Maritha J**.** Human molecular Genetics: 2025-2030.

11 - Kakoki M, Tsai y, Kim H, Hatada S, Ciavatta D, Takahashi N, Arnold L, Maeda N, Smithies O. Altering the expression in mice of genes by modifying their 3'region. Development Cell , 2004;6:597-606.