Small Dense Low-Density Lipoprotein-Cholesterol Concentrations Predict Risk for Coronary Heart Disease

The Atherosclerosis Risk in Communities (ARIC) Study

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Objective—To investigate the relationship between plasma levels of small dense low-density lipoprotein-cholesterol (sdLDL-C) and risk for incident coronary heart disease (CHD) in a prospective study among Atherosclerosis Risk in Communities (ARIC) study participants.

Approach and Results—Plasma sdLDL-C was measured in 11419 men and women of the biracial ARIC study using a newly developed homogeneous assay. A proportional hazards model was used to examine the relationship among sdLDL-C, vascular risk factors, and risk for CHD events (n=1158) for a period of ≈11 years. Plasma sdLDL-C levels were strongly correlated with an atherogenic lipid profile and were higher in patients with diabetes mellitus than non-diabetes mellitus (49.6 versus 42.3 mg/dL; P<0.0001). In a model that included established risk factors, sdLDL-C was associated with incident CHD with a hazard ratio of 1.51 (95% confidence interval, 1.21–1.88) for the highest versus the lowest quartile, respectively. Even in individuals considered to be at low cardiovascular risk based on their LDL-C levels, sdLDL-C predicted risk for incident CHD (hazard ratio, 1.61; 95% confidence interval, 1.04–2.49). Genome-wide association analyses identified genetic variants in 8 loci associated with sdLDL-C levels. These loci were in or close to genes previously associated with risk for CHD. We discovered 1 novel locus, PCSK7, for which genetic variation was significantly associated with sdLDL-C and other lipid factors.

Conclusions—sdLDL-C was associated with incident CHD in ARIC study participants. The novel association of genetic variants in PCSK7 with sdLDL-C and other lipid traits may provide new insights into the role of this gene in lipid metabolism. 

Key Words: coronary disease ■ genome-wide association study ■ triglycerides

Low-density lipoprotein-cholesterol (LDL-C) is considered one of the most important risk factors for cardiovascular disease and remains the primary target for current cardiovascular risk reduction strategies. However, many individuals with LDL-C within the normal range still develop cardiovascular disease. LDL particles are a heterogeneous population, and it has long been hypothesized that a subtraction of LDL, particularly small dense LDL (sdLDL), possesses increased atherogenic potential and thus contributes to this observation. A number of mechanisms have been proposed to explain the enhanced atherogenicity of sdLDL, including (1) a lower affinity for the LDL receptor, (2) facilitated entry into the arterial wall, (3) greater arterial retention because of increased binding to proteoglycans, and (4) greater susceptibility to oxidation. Because sdLDL particles are smaller and contain less cholesterol, increased levels of sdLDL also represent an increased number of atherogenic particles, which may not be reflected by the levels of LDL-C.

Two of the earliest and most widely used methods for LDL classification involved density and size determinations based on ultracentrifugal and nondenaturing gradient density gel electrophoresis procedures. These resulted in the division of LDL particles somewhat arbitrarily into 2 categories for clinical assessment: sdLDL and large buoyant LDL (lbLDL). These also led to the development of a 2 phenotype classification system, with phenotype A (or pattern A) characterized as individuals with a predominance of lbLDL particles and phenotype B (or pattern B) as individuals with a predominance of sdLDL particles. This classification schema has been widely used, and pattern B has been recognized as a risk marker for cardiovascular disease. More recently, the efficacy of nuclear magnetic resonance methodology to determine both particle
numbers and sizes of various lipoprotein fractions, including LDL, has been demonstrated.\textsuperscript{13} The distribution of LDL subfractions is determined by both genetic and environmental factors.\textsuperscript{13,14} Furthermore, the concentration of sdLDL is highly correlated with triglyceride level and is increased in individuals with diabetes mellitus or the metabolic syndrome.\textsuperscript{15} Therefore, it is plausible that genetic variants that affect circulating levels of sdLDL-C may also influence other lipid traits (eg, triglycerides and high-density lipoprotein-cholesterol [HDL-C]) and may aid in the identification of genes involved in the causal pathway linking atherogenic dyslipidemia characterized by sdLDL-C and coronary heart disease (CHD).

sdLDL has been found to be associated with increased risk for cardiovascular disease in cross-sectional studies\textsuperscript{16-18} and prospective observational studies.\textsuperscript{19,20} However, in most of these studies, sdLDL did not remain an independent risk predictor when adjusted for other lipid risk factors. Until recently, the methods available for the measurement of sdLDL were generally limited to nonquantitative, laborious, and highly complex techniques and, therefore, not readily adaptable to a large number of samples in a routine clinical laboratory environment. Recently, Ito et al\textsuperscript{21} developed a simple homogeneous assay adaptable to autoanalyzers for the quantification of sdLDL-C. To date, few epidemiological studies have examined whether the cholesterol content of sdLDL can predict future cardiovascular events. In the present study, we measured sdLDL-C in 11,419 men and women of the Atherosclerosis Risk in Communities (ARIC) study. These participants were followed up for a period of 11 years during which the incidence of CHD was measured. The purpose of this study was to evaluate whether sdLDL-C is a better predictor of risk for CHD than that of LDL-C and other traditional or nontraditional cardiovascular risk factors. To understand the genetic determinants of sdLDL-C and lbLDL-C better, we investigated the association of both sdLDL-C and lbLDL-C levels with genetic markers spanning the genome.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Baseline Characteristics
Key baseline (visit 4) demographics of the 11,419 ARIC participants are described in Table 1. The mean age of the study cohort was 62.8 years; 78% of study participants were white, and 56% were women. Of the study participants, 58% had a history of smoking cigarettes, 16.9% had diabetes mellitus, and 44.6% were classified with metabolic syndrome according to Adult Treatment Panel III criteria.\textsuperscript{23} Prevalent CHD was present in 972 individuals at the baseline visit, and these individuals were excluded from analyses for incident events. In the remaining 10,225 individuals (excluding 222 who had missing data on incident CHD), incident CHD developed in 1,158 participants for an average of 11 years of follow-up. The mean plasma sdLDL-C level was 43.5 mg/dL, and the mean sdLDL-C/LDL-C ratio was 0.35. Table 1 in the online-only Data Supplement shows the race- and sex-specific plasma lipid levels. Mean sdLDL-C levels were higher in whites than that in blacks and in men than women, whereas LDL-C was slightly higher in women than that in men but was not different between races. The proportion of LDL-C that was sdLDL-C was higher in whites than that in blacks and in men than women. Total cholesterol and HDL-C levels were higher in women than that in men, whereas triglyceride levels were higher in whites than that in blacks.

Association of sdLDL-C With Cardiovascular Risk Factors
Table 2 shows the means or proportions of baseline traditional risk factors and other characteristics by sdLDL-C quartiles. In general, individuals with sdLDL-C levels in the highest quartile had proatherogenic lipid profiles, were more likely to have diabetes mellitus, hypertension, and metabolic syndrome, and had higher body mass index and plasma high-sensitivity C-reactive protein (hsCRP) levels. Statin use was higher in those individuals with sdLDL-C levels in the third and fourth quartile. sdLDL-C levels were not associated with smoking status.

The correlation between sdLDL-C levels and various traditional and nontraditional cardiovascular risk factors is shown in Table 3. Strong positive correlations with sdLDL-C (|r|>0.50) were found for the lipid risk factors, such as non-HDL-C, apolipoprotein (apo) B, LDL-C, total cholesterol, and log triglycerides. Circulating levels of lipoprotein-associated phospholipase A\textsubscript{2} activity and lactate showed moderate positive correlations, and HDL-C showed a moderate negative correlation with sdLDL-C (0.25<|r|<0.50). Weaker correlations with sdLDL-C were found for fasting plasma glucose, apoAI, lbLDL-C, and log hs-CRP. High-sensitivity cardiac troponin T was not significantly correlated with sdLDL-C.

sdLDL-C Levels and Incident CHD Events
The cumulative incidence curves for CHD risk by sdLDL-C and lbLDL-C quartiles, adjusted by age, race, and sex, are shown in Figure 1. The incidence of CHD events increased proportionately during the follow-up years for participants in each consecutive quartile of sdLDL-C. In contrast, lbLDL-C
did not exhibit a concentration-dependent relationship with future incident CHD events. sdLDL-C and LDL-C levels were moderately correlated (Figure IA in the online-only Data Supplement; \( r = 0.54 \)) but often discordant. Figure IB in the online-only Data Supplement displays the prevalence and magnitude of this discordance. We examined concordant and discordant subgroups separately using a similar analysis approach as previously described by Otvos et al.\(^2\) We defined discordance as a difference of >24 percentile units (points outside the dashed lines in Figure IB in the online-only Data Supplement). Figure II in the online-only Data Supplement shows the cumulative incidence curves for CHD risk for the subgroup with sdLDL-C<LDL-C discordance when compared with the concordant and the discordant sdLDL-C<LDL-C subgroups. The sdLDL-C<LDL-C discordant subgroup showed the highest CHD risk when compared with the concordant and discordant sdLDL-C<LDL-C subgroups.

We used proportional hazards regression analyses to investigate the association of incident CHD with baseline levels of sdLDL-C and LDL-C modeled in quartiles, using quartile 1 as the referent group (Table 4). In the basic model adjusted for age, race, and sex (model 1), individuals with sdLDL-C levels in the highest quartile had a 2-fold higher risk for incident CHD when compared with those in the lowest quartile (hazard ratio [HR], 2.00; 95\% confidence interval [CI], 1.69–2.37). After additional adjustment for smoking, body mass index, hypertension, HDL-C, log triglycerides, lipid-lowering medications, diabetes mellitus, diabetes mellitus medications, and log hs-CRP (model 2), risk for incident CHD was attenuated but remained significant (HR, 1.51; 95\% CI, 1.21–1.88). sdLDL-C was not significantly associated with risk for incident CHD after further adjustment for other lipid risk factors, such as LDL-C, apo B, and total cholesterol. This may be, in part, because of over adjustment of the multivariable model and is not surprising given the strong correlations of sdLDL-C with these lipid risk factors. In comparison, individuals with LDL-C levels in the highest quartile had a 56\% and 68\% higher risk for incident CHD when compared with those in the lowest quartile (HR, 1.56; 95\% CI, 1.32–1.83 and HR, 1.68; 95\% CI, 1.42–1.99) in the basic model (model 1) and more fully adjusted model (model 2), respectively.

We further investigated the association of sdLDL-C with risk for incident CHD in participants stratified by LDL-C risk categories (ie, LDL-C<100 mg/dL and LDL-C \( \geq \) 100 mg/dL). In these analyses, we used a multivariable model (adjusting for age, sex, race, ever smoking, body mass index, hypertension, diabetes mellitus, diabetes mellitus medication, and log hs-CRP), with quartile 1 for sdLDL-C in the LDL-C<100 mg/dL category as the referent group.
mg/dL risk category as the referent group. Even in individuals with LDL-C levels <100 mg/dL, sdLDL-C was predictive of CHD risk across increasing sdLDL-C quartiles (Figure 2). Participants with LDL-C <100 mg/dL and sdLDL-C levels in the fourth quartile had a 61% increase in risk for incident CHD (HR, 1.61; 95% CI, 1.04–2.49) when compared with individuals with sdLDL-C levels in the first quartile. In comparison, participants with LDL-C ≥ 100 mg/dL and sdLDL-C levels in the fourth quartile had an 86% increase in risk for incident CHD (HR, 1.86; 95% CI, 1.48–2.33) when compared with those in the same referent group.

In additional analyses, we examined the effects on CHD risk of sdLDL-C discordance among ARIC participants with low LDL-C (<100 mg/dL; <25th percentile) or equivalently low sdLDL-C (<27.8 mg/dL; <25th percentile; Figure 3). The cumulative incidence of CHD events was higher among individuals with low LDL-C but discordantly higher sdLDL-C (10.9%) when compared with individuals with low sdLDL-C but discordantly higher LDL-C (7.9%). Not surprisingly, the cumulative incidence of CHD events was highest among individuals with concordantly higher levels of LDL-C and sdLDL-C (12.7%) and lowest among individuals with concordantly lower levels of LDL-C and sdLDL-C (7.6%).

Genome-Wide Association Study of sdLDL-C
We performed genome-wide association study (GWAS) analyses of sdLDL-C, and Table 5 summarizes our primary findings. In total, 127 single-nucleotide polymorphisms (SNPs) were significantly associated with sdLDL-C (P<5×10⁻⁸). These SNPs were clustered at 8 different loci on chromosomes 1, 2, 7, 8, 11, and 19 and were located within 14 different genes (or gene clusters). With the exception of PCSK7, genetic variants within all of these genes have previously been found to be related to pathways involved in lipid metabolism and vascular inflammation (www.genome.gov).

Association of PCSK7 SNP rs508487 Genotype With Circulating Lipids and CHD
A novel finding from the current GWAS analysis was the significant association of sdLDL-C with SNP rs508487 (at locus 11q23–q24) located in the PCSK7 gene. We investigated the effect of rs508487 genotype on circulating lipid levels (Table 6).

![Figure 1](http://atvb.ahajournals.org/)

**Figure 1.** Cumulative incidence curves for risk of coronary heart disease (CHD) by small dense low-density lipoprotein-cholesterol (sdLDL-C) and large buoyant LDL-C (lbLDL-C) quartiles, adjusted for age, race, and sex.
the association between PCSK7 rs508487 and sdLDLC in 6069 individuals homozygous for the wild-type allele at APOA5 rs662799, and the results were attenuated a bit but still nominally statistically significant (P=0.0027).

We investigated the relationship between PCSK7 SNP rs508487 genotype and CHD in the ARIC study and did not observe a significant association. However, the power for this particular analysis was limited because of the fact that the number of ARIC CHD cases with 1 or 2 minor alleles at this SNP was low. Therefore, we examined the association of rs508487 with 40 260 CHD cases from the CARDIoGRAM study and found that rs508487 was significantly associated with CHD (odds ratio, 1.13; 95% CI, 1.06–1.21; P=0.00017).

We next analyzed rare variants on the Illumina exome chip designated as nonsynonymous, splicing, or stop gain in the PCSK7 gene for association with sdLDLC. We found a total of 7 nonsynonymous PCSK7 variants among white ARIC participants, resulting in amino acid substitutions in the wild-type PCSK7 protein. Because these variants had minor allele frequencies <1%, we analyzed them collectively for their effect on sdLDLC. Individuals who were carriers of any of the rare PCSK7 variants had a significant increase in circulating levels of sdLDLC (6.7 mg/dL; P=0.012) and triglycerides (ln(TG) =0.145; P=0.043) when compared with noncarriers (Table 7).

**Figure 2.** Adjusted hazard ratios (HRs) for incident coronary heart disease by small dense low-density lipoprotein-cholesterol (sdLDLC) quartiles stratified by LDL-C risk categories, adjusted for age, sex, and race, smoking, body mass index, hypertension, diabetes mellitus, diabetes mellitus medications, and log(high-sensitivity C-reactive protein). CI indicates confidence interval.

**Table 4.** Hazard Ratio (95% Confidence Interval) for Incident Coronary Heart Disease by sdLDLC and LDL-C Quartiles

<table>
<thead>
<tr>
<th>Quartile of sdLDLC-C†</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>P Value§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model 1†</td>
<td>1.19 (0.99–1.43)</td>
<td>1.44 (1.21–1.72)</td>
<td>2.00 (1.69–2.37)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Model 2‡</td>
<td>1.10 (0.90–1.33)</td>
<td>1.21 (0.99–1.48)</td>
<td>1.51 (1.21–1.88)</td>
<td>0.0008</td>
</tr>
</tbody>
</table>

sdLDLC indicates small dense low-density lipoprotein-cholesterol.

§Adjusted for age, sex, and race.

†Adjusted for model 1 variables+smoking, body mass index, hypertension, high-density lipoprotein-cholesterol, log(triglycerides), lipid-lowering medications, diabetes mellitus, diabetes mellitus medications, and log(high-sensitivity C-reactive protein).

**Discussion**

In the current study, we investigated the relationship between plasma levels of sdLDLC and risk of incident CHD in the predominantly biracial ARIC study cohort using a newly developed automated homogeneous sdLDLC assay. Elevated plasma sdLDLC levels were associated with increased risk of incident CHD in a multivariable model (HR, 1.51; 95% CI, 1.21–1.88) and even in individuals considered to be at low cardiovascular risk based on their LDL-C levels, sdLDLC predicted risk for incident CHD (HR, 1.61; 95% CI, 1.04–2.49). Using GWA analyses, we discovered 1 novel locus, PCSK7, for which genetic variation was significantly associated with sdLDLC levels and other lipid traits. Subsequent examination in the CARDioGRAM study showed a significant association of the PCSK7 SNP rs508487 with CHD.

**sdLDLC and Risk for Incident CHD**

Among ARIC participants, the mean baseline plasma sdLDLC level was 43.5 mg/dL, which represented 35% of the total LDL-C concentration. sdLDLC and sdLDLC/LDL-C ratio were higher in whites than in blacks and higher in men than in women.

Our findings related to circulating sdLDLC levels seem in general agreement with a report from the Framingham Offspring Study, which showed that men had higher sdLDLC levels and a higher percentage of LDL-C as sdLDLC when compared with women.23 Although we found higher mean sdLDLC and sdLDLC/LDL-C ratio overall than those reported in the Framingham Offspring Study, these differences may be, in part, because of differences in study populations (eg, the ARIC cohort has a higher prevalence of obesity and metabolic syndrome than the Framingham Offspring Study) and sdLDLC assay methodologies.

Plasma levels of sdLDLC were adversely associated with cardiovascular lipid risk factors, a finding consistent with...
previous reports showing a correlation of sdLDL with an atherogenic lipid profile. We also found significant correlations between sdLDL-C and nonlipid risk factors, such as fasting glucose and lactate levels. Even though sdLDL-C was associated with diabetes mellitus and metabolic syndrome in previous studies, we report a remarkable increase in prevalence of metabolic syndrome among individuals with sdLDL-C levels in the highest quartile (73%) when compared with those in the lowest quartile (23%). sdLDL-C was also correlated with inflammatory markers, such as lipoprotein-associated phospholipase A2 activity and hs-CRP.

During the 11-year follow-up period of this study, 1158 (11.3%) participants developed CHD. The cumulative incidence curves clearly illustrate the direct relation between sdLDL-C levels and CHD risk, whereas we did not find a similar relation between lbLDL-C and CHD. These results suggest that the sdLDL subfraction is a major contributor to the risk for incident CHD that is associated with LDL-C. Circulating levels of sdLDL-C were significantly associated with increased risk for CHD in a model adjusted for age, sex, and race and in a more fully adjusted model that also included smoking, body mass index, hypertension, HDL-C, triglycerides, lipid-lowering medications, diabetes mellitus, diabetes mellitus medications, and hs-CRP. However, sdLDL-C was not an independent predictor of incident CHD when we further adjusted for other lipid risk factors, such as LDL-C, apo B, and total cholesterol, which is not surprising given the strong correlations of sdLDL-C with these other lipid risk factors, and our results are in agreement with previous studies reporting that sdLDL was not an independent predictor of cardiovascular disease.26-28 Interestingly, sdLDL-C showed predictive power for CHD risk even in individuals with optimal LDL-C levels as defined in the current guidelines (<100 mg/dL).23 Several investigators have emphasized that the number of particles as measured by nuclear magnetic resonance is more important for assessment of cardiovascular risk than LDL subclass, LDL particle size, or LDL-C concentration.29,30 Because sdLDL particles contain less cholesterol than lbLDL particles, there are more sdLDL particles than lbLDL particles at a given LDL-C concentration. Whether the total number of particles or the cholesterol payload per particle is more important to cardiovascular risk remains a topic of discussion. However, it is important to note that the particle number theory does not take into account the accumulating evidence pointing to different atherogenic properties of LDL subclasses. A limitation of this study is the fact that we did not have particle number information available and thus we were not able to address this question specifically.

### Genetics of sdLDL-C

GWAS analysis identified a large number of SNPs clustered at 8 different loci on chromosomes 1, 2, 7, 8, 11, and 19 that were significantly associated with sdLDL-C. With the exception of PCSK7, genetic variants located in all the genes associated with sdLDL-C were significant after adjustment for other lipid risk factors, such as LDL-C, apo B, and total cholesterol. However, sdLDL-C was not an independent predictor of incident CHD when further adjusted for other lipid risk factors, such as LDL-C, apo B, and total cholesterol, which is not surprising given the strong correlations of sdLDL-C with these other lipid risk factors, and our results are in agreement with previous studies reporting that sdLDL was not an independent predictor of cardiovascular disease.26-28 Interestingly, sdLDL-C showed predictive power for CHD risk even in individuals with optimal LDL-C levels as defined in the current guidelines (<100 mg/dL).23 Several investigators have emphasized that the number of particles as measured by nuclear magnetic resonance is more important for assessment of cardiovascular risk than LDL subclass, LDL particle size, or LDL-C concentration.29,30 Because sdLDL particles contain less cholesterol than lbLDL particles, there are more sdLDL particles than lbLDL particles at a given LDL-C concentration. Whether the total number of particles or the cholesterol payload per particle is more important to cardiovascular risk remains a topic of discussion. However, it is important to note that the particle number theory does not take into account the accumulating evidence pointing to different atherogenic properties of LDL subclasses. A limitation of this study is the fact that we did not have particle number information available and thus we were not able to address this question specifically.

### Table 5. Association of the Top SNPs With sdLDL-C

<table>
<thead>
<tr>
<th>SNP</th>
<th>Location</th>
<th>Chromosome</th>
<th>No. of Sign SNPs</th>
<th>Coded Allele</th>
<th>Allele Frequency</th>
<th>n</th>
<th>β</th>
<th>SE(β)</th>
<th>P Value</th>
<th>Gene</th>
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</thead>
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<td>C</td>
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<td>6979</td>
<td>-5.70008</td>
<td>0.51138</td>
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<td>APOA5/A4/C3/A1</td>
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<tr>
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<td>6</td>
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<td>0.49027</td>
<td>9.77×10⁻²⁹</td>
<td>APOE/C1/C4/C2</td>
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<td>3</td>
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<td>6979</td>
<td>3.45306</td>
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<td>0.46476</td>
<td>4.60×10⁻¹⁰</td>
<td>BAZ1B</td>
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sdLDL-C indicates small dense low-density lipoprotein-cholesterol; and SNP, single-nucleotide polymorphism.

### Table 6. Association of PCSK7 Single-Nucleotide Polymorphism rs508487 Genotype With Circulating Lipids

<table>
<thead>
<tr>
<th>Lipid, mg/dL (mean±SE)</th>
<th>Genotype</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC</td>
<td>CT</td>
</tr>
<tr>
<td>sdLDL-C</td>
<td>44.3±20.6</td>
<td>48.5±21.4</td>
</tr>
<tr>
<td>lbLDL-C</td>
<td>78.7±27.5</td>
<td>75.5±29.7</td>
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<td>sdLDL-C/LDL-C</td>
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<td>0.40±0.16</td>
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<td>LDL-C</td>
<td>123.0±32.8</td>
<td>124.0±32.6</td>
</tr>
<tr>
<td>HDL-C</td>
<td>50.2±16.4</td>
<td>48.6±16.1</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>201.5±35.6</td>
<td>205.0±35.0</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>141.5±67.5</td>
<td>162.1±77.5</td>
</tr>
</tbody>
</table>

HDL-C indicates high-density lipoprotein-cholesterol; lbLDL-C, large buoyant low-density lipoprotein-cholesterol; and sdLDL-C, small dense low-density lipoprotein-cholesterol.
with sdLDL-C levels have been reported previously in GWAS of blood lipid levels.\textsuperscript{28} Our GWAS findings are in general agreement with an earlier report by Chasman et al\textsuperscript{29} who used a comprehensive GWAS analysis to identify largely similar loci that affect the nuclear magnetic resonance–based measures of concentration and size of LDL, HDL, and very LDL particles in women. Although the study by Chasman et al\textsuperscript{29} did not find a significant association with LDL particle size or concentration at the PCSK7 locus, this apparent discrepancy may be because of notable differences between the 2 studies, such as differences in methodologies to measure lipoprotein fractions, genotyping methods, and study populations. Indeed, a recent report from the Multi-Ethnic Study of Atherosclerosis (MESA) compared the identical automated assay of sdLDL-C as was used in our study to nuclear magnetic resonance–derived small LDL concentrations with regards to risk prediction for incident CHD.\textsuperscript{30} The authors showed that the newly automated assay of sdLDL-C identified the risk of CHD, whereas the nuclear magnetic resonance–derived small LDL concentrations did not convey a significant risk of CHD in women. Although the study by Chasman et al\textsuperscript{29} did not find a significant association with LDL particle size or concentration at the PCSK7 locus, this apparent discrepancy may be because of notable differences between the 2 studies, such as differences in methodologies to measure lipoprotein fractions, genotyping methods, and study populations. Indeed, a recent report from the Multi-Ethnic Study of Atherosclerosis (MESA) compared the identical automated assay of sdLDL-C as was used in our study to nuclear magnetic resonance–derived small LDL concentrations with regards to risk prediction for incident CHD.\textsuperscript{30} The authors showed that the newly automated assay of sdLDL-C identified the risk of CHD, whereas the nuclear magnetic resonance–derived small LDL concentrations did not convey a significant risk of CHD in women. Therefore, if these 2 different methodologies show different associations with cardiovascular risk, it is plausible that they may also lead to different GWAS findings.

Genetic variants within a number of the genes associated with sdLDL-C in our study have previously been found to be associated with increased risk for cardiovascular disease in meta-analyses of GWA studies.\textsuperscript{31,32} SNP rs4420638, which is located in the \textit{APOE-APOC1-APOC4-APOC2} gene cluster, was also associated with lipoprotein-associated phospholipase A2 activity and CHD in a meta-analysis of GWA studies from 5 community-based studies.\textsuperscript{33} In addition, we have previously shown an association of the SNP rs780094 in \textit{GCKR} with metabolic syndrome prevalence and incident diabetes mellitus in the ARIC study.\textsuperscript{34} rs780094 was also significantly associated with sdLDL-C (\(P=4.08\times10^{-12}\)) in our current study.

Our findings have important implications in light of recent observations from Mendelian randomization studies investigating genetic determinants of HDL-C levels and risk for incident CHD. Unlike data from human Mendelian diseases, which support a causal role for LDL-C in risk for CHD,\textsuperscript{35,36} evidence for a causal role of HDL-C from Mendelian randomization studies is inconsistent and complicated by the fact that most SNPs associated with HDL-C levels affect multiple lipid traits. Voight et al\textsuperscript{37} recently showed that a genetic risk score consisting of 14 SNPs exclusively associated with HDL-C was not associated with risk for myocardial infarction, in contrast to a genetic risk score for LDL-C. These investigators had previously shown that a number of SNPs associated with HDL-C were also associated with other lipid traits, such as triglycerides and LDL-C.\textsuperscript{38} The SNPs associated with HDL-C that were most strongly associated with increased risk for myocardial infarction and that influenced other lipid traits were located in or near the \textit{APOA5-APOA4-APOC3-APOA1}, \textit{TRIB1}, and \textit{LPL} genes or gene clusters. In the current study, we showed that these genes are also associated with sdLDL-C or sdLDL-C/LDL-C ratio (Table II in the online-only Data Supplement).

### Association of PCSK7 SNP rs508487 Genotype With Circulating Lipids

A novel finding from the current GWAS analysis is the significant association of sdLDL-C with SNP rs508487 (at locus 11q23-q24), located in the PCSK7 gene. Investigation of the effect of rs508487 genotype on circulating lipid levels showed that each copy of the minor allele at this SNP raised sdLDL-C by \(\approx 4\) mg/dL. It is important to note that genetic variation at the PCSK7 locus was not associated with LDL-C levels in our study. Because LDL-C is a more commonly used lipid measurement, it is plausible that genetic variations in the PCSK7 gene have not been associated with circulating lipids in previous GWAS studies.

\textit{PCSK7} encodes subtilisin-like/kexin proprotein convertase type 7 (PCSK7), a calcium-dependent serine endoprotease. PCSK7 has previously been implicated as a mediator of adipogenesis\textsuperscript{39} and in the processing of VEGF-D, a critical step for binding of the angiogenic receptor VEGFR-2.\textsuperscript{40} Furthermore, recent data show that internalization of PCSK7 from the plasma membrane is mediated by clathrin-coated vesicles,\textsuperscript{41} which are also implicated in the internalization of other cellular receptors, such as the LDL receptor and various scavenger receptors. Although the physiological role of PCSK7 is not clearly understood, it is plausible that PCSK7 could be involved in the processing of LDL and scavenger receptors, thereby modulating circulating lipid levels. Alternatively, recent studies suggest a potential role of protein convertases, including PCSK7, in lipid metabolism through proteolytic activation of angiopoietins and proteolytic inactivation of lipases.\textsuperscript{42,43} In contrast to PCSK9, another member of the proprotein convertase family, to our knowledge PCSK7 has not been previously associated with cardiovascular lipid risk factors in other GWA studies. However, we should be cautious in the interpretation of our findings about the association of PCSK7 genotype with circulating lipids. A limitation of our study is that we did not measure protein or mRNA levels of PCSK7. Furthermore, the PCSK7 SNP rs508487 is in close proximity to the \textit{APOA5-APOA4-APOC3-APOA1} gene cluster, which has also been shown to affect circulating triglyceride levels. However, our exome chip data show that rare variants in the PCSK7 gene, which lead to amino acid substitutions in the PCSK7 protein, are associated with sdLDL-C and other lipid traits. Additional in vitro or animal studies using transgenic or PCSK7-knockout mouse models are needed to investigate the potential role of PCSK7 in lipid metabolism. Our findings also highlight the important issue of pleiotropy because PCSK7 was associated with circulating levels of sdLDL-C, triglycerides, and HDL-C.

### Conclusions

In summary, our results showed that sdLDL-C was highly correlated with an atherogenic lipid profile and, in contrast to
ldL-C, predicted future CHD events in ARIC participants. Furthermore, sLDL-C predicted risk for incident CHD even in individuals who would be considered at low cardiovascular risk based on their LDL-C level. This new homogenous sLDL-C assay could be readily implemented in most routine clinical chemistry laboratories, provided that its clinical value can be confirmed in future studies. GWAS analysis identified significant associations of sLDL-C with genetic variants in 14 different genes, all but 1 of which have been previously linked to cardiovascular disease risk. Our GWAS findings, together with findings from previous studies showing genetic variants in the same genes associated with other lipid traits, highlight the importance of pleiotropy in the development of cardiovascular disease. The novel finding of a significant association of sLDL-C with genetic variants in PCSK7, a member of the subtilisin-like/kexin propertase convertase family, provides new insights into the role of this gene in lipid metabolism.

Acknowledgments

We thank the staff and participants of the Atherosclerosis Risk in Communities (ARIC) study for their important contributions.

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Disclosures

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27. Mora S, Szkl M, Otvos JD, Greenland P, Patsy BM, Goff DC Jr, O’Leary DH, Saad MF, Tsai MY, Sharrett AR. LDL particle sub classes, LDL
Low-density lipoprotein-cholesterol (LDL-C) is considered one of the most important risk factors for cardiovascular disease and remains the primary target for current cardiovascular risk reduction strategies. LDL particles are a heterogeneous population, and it has long been hypothesized that a subtraction of LDL, small dense LDL, possesses atherogenic potential. In the current study, we investigated the relationship between plasma levels of small dense LDL-C and risk of incident coronary heart disease in the biracial Atherosclerosis Risk in Communities (ARIC) study cohort. Elevated plasma small dense LDL-C levels were associated with increased risk of incident coronary heart disease, even in individuals considered to be at low cardiovascular risk based on their LDL-C levels. Using genome-wide association analyses, we discovered 1 novel locus, PCSK7, for which genetic variation was significantly associated with small dense LDL-C levels and other lipid traits. Together these findings provide new insights into the role of the PCSK7 gene in lipid metabolism and risk of cardiovascular disease.
Small Dense Low-Density Lipoprotein-Cholesterol Concentrations Predict Risk for Coronary Heart Disease: The Atherosclerosis Risk in Communities (ARIC) Study
Ron C. Hoogeveen, John W. Gaubatz, Wensheng Sun, Rhiannon C. Dodge, Jacy R. Crosby, Jennifer Jiang, David Couper, Salim S. Virani, Sekar Kathiresan, Eric Boerwinkle and Christie M. Ballantyne

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MATERIAL AND METHODS

Study Design and Study Participants

The ARIC study is a prospective investigation of cardiovascular disease incidence involving 15,792 men and women aged 45 to 64 years and recruited from four U.S. communities in 1987–1989. Participants underwent a baseline exam and up to 4 follow-up visits. A detailed description of the ARIC study design and methods has been published elsewhere. The current study was conducted among individuals who participated in ARIC study visit 4 (1996-1998). Of the 11,656 eligible individuals who participated in visit 4, we excluded those without sdLDL-C data (n=168), with self-reported race being neither white nor black (n=31), and black race at centers in Minneapolis or Washington County (n=38). For incident CHD analyses, individuals with prevalent CHD at visit 4 (n=972), or those with missing covariate data for the multivariable models (n=565) were excluded, resulting in 9,882 individuals who were included in our final analysis. Prevalent CHD was defined as self-reported myocardial infarction before visit 1 or silent myocardial infarction (diagnosed by electrocardiographic changes), validated myocardial infarction, or revascularization between visits 1 and 4.

The ascertainment procedure for incident CHD events has been described previously. Briefly, incident CHD was defined as those participants with hospitalized myocardial infarction, fatal CHD, or cardiac procedure by 2008.

Participant Examination

Medical history, demographic data, anthropometric data, blood pressure measurements (ARIC Manual 11 visit 4, NHLBI 1997), fasting glucose, and fasting lipids (ARIC Manual 8, NHLBI 1994) obtained during visit 4 were used for this analysis. Cigarette smoking and the use of antihypertensive and lipid-lowering medications were ascertained from a standardized questionnaire.
Hypertension was defined as systolic blood pressure ≥140 mm Hg, diastolic blood pressure ≥90 mm Hg, prior physician diagnosis of hypertension, or use of antihypertensive medication during the previous 2 weeks. Diabetes mellitus was defined as a fasting glucose level ≥126 mg/dL, a nonfasting glucose level ≥200 mg/dL, or a self-reported history of physician-diagnosed diabetes or treatment for diabetes. The study was approved by the institutional review committees of all participating centers, and all participants provided informed consent.

**Laboratory Analyses**

Plasma total cholesterol, triglycerides, high-density lipoprotein cholesterol (HDL-C) were measured using enzymatic methods; low-density lipoprotein (LDL) cholesterol was calculated according to the Friedewald formula. Large buoyant LDL cholesterol (lbLDL-C) was estimated by subtracting the sdLDL-C concentration from the LDL-C concentration. Non-HDL-C was calculated by subtracting the HDL-C concentration from the total cholesterol concentration. The fraction of LDL-C which was sdLDL-C was calculated by dividing the sdLDL-C concentration by the LDL-C concentration. Plasma apolipoproteins AI and B and high-sensitivity C-reactive protein (hs-CRP) were measured by an immunonephelometric assay using a BNII nephelometer (Siemens Healthcare Diagnostics, Deerfield, IL).

A homogeneous assay method was used for the direct measurement of sdLDL-C in plasma (sd-LDL-EX “Seiken”, Denka Seiken, Tokyo, Japan) on a Hitachi 917 automated chemistry analyzer. This method has been previously validated and shown to be in good agreement with the ultracentrifugal method used to isolate LDL in the 1.044–1.063 g/ml density range used by many investigators for sdLDL, with an $r^2=0.91$. The intra-assay and inter-assay coefficients of variation for the sdLDL-C assay were 1.3% and 3.1%, respectively. The reliability coefficient for the sdLDL-C assay based on 435 blinded quality control replicates was 0.92. It is important to note that the lbLDL-C fraction may include intermediate-density lipoprotein (IDL)
cholesterol since we did not use an ultracentrifugation method to isolate this specific lbLDL-C fraction.

**Genotyping**

Genome-wide genotyping of single-nucleotide polymorphisms (SNPs) was performed using the Affymetrix Genome-Wide Human SNP Array 6.0 (Santa Clara, CA). Study participants who refused DNA testing, had high missing rates, suspected contaminated samples, samples with genotype mismatch with 47 previously genotyped SNPs, and genetic outliers based on identity-by-state statistics and EIGENSTRAT principal components analysis were excluded. Additionally, monomorphic SNPs, SNPs with no chromosome location, and SNPs with call rate <95%, minor allele frequency <1%, or Hardy–Weinberg equilibrium p<10\(^{-6}\) were also excluded. The Affymetrix 6.0 genotypes and a cosmopolitan set of HapMap haplotypes were used to impute 2.4 million autosomal SNPs. Imputation results were summarized as an allele dosage, which was defined as the expected number of copies of the minor allele at each SNP. We applied an *a priori* threshold of 5.0\(\times 10^{-8}\) for statistical significance for these genome-wide association analyses. When more than 1 genome-wide significant SNP clustered at a locus, we took the SNP with the smallest P value as the lead SNP.

Genome-wide association study (GWAS) analyses were adjusted for age and gender, using a co-dominant model. For the GWAS, linear regression analyses of sdLDL-C, lbLDL-C, and the ratio of sdLDL-C to LDL-C were carried out using PLINK (version 1.07) and ProbABEL, and Cox proportional hazards modeling was used to test for an independent association of select genetic variants and the presence of incident CHD. The association between the genome-wide significant SNP rs508487 located in the PCSK7 gene and CHD was examined from results in 40,260 cases and 60,790 controls from the Coronary Artery Disease Genome-Wide Replication And Meta-Analysis (CARDIoGRAM) Study.\(^5\) Although details differed among
the contributing studies in CARDIoGRAM, the definition of CHD included clinically defined myocardial infarction or angiographically accessed coronary artery disease.

Using ARIC data from the CHARGE (Cohorts for Heart and Aging Research in Genome Epidemiology) Exome Chip project, we analyzed rare variants designated as nonsynonymous, splicing, or stop gain in the PCSK7 gene for association with sdLDL-C as previously described.6 These variants were first tested individually for association with sdLDL-C using linear regression. Since these variants had minor allele frequencies below 1%, they were also tested collectively in two separate gene-based tests. The first gene-based test was the T1 burden test used to detect an association between variation in each gene and sdLDL-C. This test is the most powerful when all variants have the same direction of effect on the phenotype. The second gene-based was the SKAT test which allowed for different directions of effect between the variants included. All analyses were adjusted for age and gender.

**Statistical Analyses**

The distribution of sdLDL-C and all other clinically relevant continuous variables measured in our analysis population were evaluated to assess normality. For this analysis, we modeled sdLDL-C both as a continuous and categorical variable. As a categorical variable, quartile measures were used as cut-points to obtain four separate groups. The cut-points were obtained from the distribution of sdLDL-C in the whole analysis population (25th, 50th, and 75th percentile values were 28.0, 39.7, and 54.7 mg/dL, respectively). Means or proportions of demographic characteristics and traditional cardiovascular risk factors of the study participants were reported by sdLDL-C quartiles. The p-values for trends were evaluated with linear or logistic regression using quartile number adjusted for age, race, and gender. Pearson’s correlation coefficient was used to assess the correlation of sdLDL-C and traditional or novel cardiovascular risk factors. Triglycerides and hs-CRP were log-transformed to account for their non-Gaussian distributions. Associations between sdLDL-C and incident CHD were determined using Cox proportional
hazards modeling, in both unadjusted and adjusted models. The basic model (Model 1) adjusted for age, gender, and race as potential confounders. Model 2 was additionally adjusted for smoking status (current versus not current), body mass index (BMI), hypertension, antihypertensive medication use, HDL-C, log triglycerides, lipid-lowering medication use, presence of diabetes mellitus (defined as a fasting glucose level ≥126 mg/dL, a nonfasting glucose level ≥200 mg/dL, or a self-reported history of physician-diagnosed diabetes), diabetes medication use, and log hs-CRP. In all models, the 2nd, 3rd and 4th quartiles were compared to the 1st quartile (the referent group). Statistical analysis was performed using SAS version 9.2 (SAS Institute Inc., Cary, NC) and STATA version 11 (StataCorp LP, College Station, TX). All tests were 2-sided with a p-value <0.05 considered significant.
References


Supplemental Table I: Race- and gender-specific lipid levels in the ARIC study cohort

<table>
<thead>
<tr>
<th>Lipid fraction</th>
<th>Caucasian Women (N=4763)</th>
<th>Caucasian Men (N=4141)</th>
<th>African American Women (N=1622)</th>
<th>African American Men (N=893)</th>
<th>P-value† by race</th>
<th>P-value† by gender</th>
</tr>
</thead>
<tbody>
<tr>
<td>sdLDL-C, mg/dL</td>
<td>45.07 (.3206)</td>
<td>45.35 (.3070)</td>
<td>36.57 (.4323)</td>
<td>38.84 (.6268)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>lbLDL-C, mg/dL</td>
<td>78.79 (.4182)</td>
<td>75.31 (.4281)</td>
<td>87.85 (.7210)</td>
<td>83.87 (.9142)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LDL-C, mg/dL</td>
<td>123.60 (.4985)</td>
<td>120.81 (.4846)</td>
<td>124.31 (.9038)</td>
<td>122.69 (.1831)</td>
<td>0.4790</td>
<td>0.1100</td>
</tr>
<tr>
<td>sdLDL-C/LDL-C ratio</td>
<td>.3631 (.0022)</td>
<td>.3765 (.0024)</td>
<td>.2961 (.0032)</td>
<td>.3166 (.0043)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>209.41 (.5306)</td>
<td>191.71 (.5350)</td>
<td>203.24 (.9575)</td>
<td>193.29 (1.2391)</td>
<td>&lt;0.0001</td>
<td>0.2197</td>
</tr>
<tr>
<td>HDL-C, mg/dL</td>
<td>55.472 (.2407)</td>
<td>41.695 (.1933)</td>
<td>56.105 (.4073)</td>
<td>47.952 (.5391)</td>
<td>0.1832</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>153.78 (.3302)</td>
<td>150.04 (.4051)</td>
<td>114.81 (.5708)</td>
<td>115.46 (2.2402)</td>
<td>&lt;0.0001</td>
<td>0.0532</td>
</tr>
</tbody>
</table>

*Data presented as mean (standard error of the mean).
†P-value for test of differences in means, and p-value by race and gender.
## Supplemental Table II: Association of the top SNPs in 8 loci with sdLDL-C/LDL-C ratio

<table>
<thead>
<tr>
<th>SNP</th>
<th>Location</th>
<th>Chromosome</th>
<th># Sign SNPs</th>
<th>Coded Allele</th>
<th>Allele Frequency</th>
<th>N</th>
<th>Beta</th>
<th>SEBeta</th>
<th>P</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs964184</td>
<td>11q23.3</td>
<td>11</td>
<td>42</td>
<td>C</td>
<td>0.8588</td>
<td>6860</td>
<td>-0.04646</td>
<td>0.003705</td>
<td>4.40×10⁻³⁶</td>
<td>APOA5/A4/C3/A1/ZNF259/BUD13</td>
</tr>
<tr>
<td>rs4420638</td>
<td>19q13.32</td>
<td>19</td>
<td>4</td>
<td>A</td>
<td>0.8264</td>
<td>6860</td>
<td>-0.03217</td>
<td>0.003545</td>
<td>1.13×10⁻¹⁹</td>
<td>APOE/C1/C4/C2/TOMM40</td>
</tr>
<tr>
<td>rs1260326</td>
<td>2p23.3</td>
<td>2</td>
<td>7</td>
<td>C</td>
<td>0.5856</td>
<td>6860</td>
<td>-0.02206</td>
<td>0.002637</td>
<td>5.98×10⁻¹⁷</td>
<td>GCKR/IFT172/ZNF51</td>
</tr>
<tr>
<td>rs2980855</td>
<td>8q24.13</td>
<td>8</td>
<td>32</td>
<td>C</td>
<td>0.4682</td>
<td>6860</td>
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<td>0.002547</td>
<td>1.28×10⁻¹⁴</td>
<td>TRIB1</td>
</tr>
<tr>
<td>rs13244268</td>
<td>7q11.23</td>
<td>7</td>
<td>22</td>
<td>C</td>
<td>0.1232</td>
<td>6860</td>
<td>-0.02587</td>
<td>0.003935</td>
<td>4.87×10⁻¹¹</td>
<td>BAZ1B/MLXIPL/TBL2/BCL7B/VPS37D</td>
</tr>
<tr>
<td>rs508487</td>
<td>11q23-q24</td>
<td>11</td>
<td>2</td>
<td>C</td>
<td>0.9402</td>
<td>6860</td>
<td>-0.04084</td>
<td>0.006312</td>
<td>9.75×10⁻¹¹</td>
<td>PCSK7</td>
</tr>
<tr>
<td>rs995000</td>
<td>1p31.3</td>
<td>1</td>
<td>70</td>
<td>C</td>
<td>0.6687</td>
<td>6860</td>
<td>0.015624</td>
<td>0.002702</td>
<td>7.32×10⁻⁰⁹</td>
<td>DOCK7/ANGPTL3/USP1</td>
</tr>
<tr>
<td>rs7586601</td>
<td>2p23</td>
<td>2</td>
<td>1</td>
<td>A</td>
<td>0.556</td>
<td>6860</td>
<td>0.014932</td>
<td>0.002615</td>
<td>1.12×10⁻⁰⁸</td>
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<tr>
<td>rs11208046</td>
<td>1p31.3</td>
<td>1</td>
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<td>C</td>
<td>0.5658</td>
<td>6860</td>
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<td>0.002605</td>
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<tr>
<td>rs12678919</td>
<td>8p22</td>
<td>8</td>
<td>3</td>
<td>A</td>
<td>0.893</td>
<td>6860</td>
<td>0.021005</td>
<td>0.004236</td>
<td>7.12×10⁻⁰⁷</td>
<td>LPL</td>
</tr>
</tbody>
</table>
**Supplemental Figure I: A)** Correlation of LDL-C and sdLDL-C concentrations among 11234 ARIC participants. **B)** Relation of LDL-C and sdLDL-C levels given in percentile units. The dashed lines bracket concordant LDL-C and sdLDL-C values defined as those within ±24 percentile units.
**Supplemental Figure II:** Cumulative incidence of cardiovascular events in sub-groups with concordant or discordant levels of LDL-C and sdLDL-C from proportional hazards models adjusted for age, gender, and race.