

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 11 419 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. (*Arterioscler Thromb Vasc Biol.* 2014;34:1069-1077.)

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.^{1,2} However, many individuals with LDL-C within the normal range still develop cardiovascular disease.^{3,4} LDL particles are a heterogeneous population,⁵ and it has long been hypothesized that a subfraction 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,^{6–10} 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.

See accompanying editorial on page 959

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

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Nonstandard Abbreviations and Acronyms

apo	apolipoprotein
ARIC	Atherosclerosis Risk in Communities
CHD	coronary heart disease
CI	confidence interval
GWAS	genome-wide association study
HDL-C	high-density lipoprotein-cholesterol
HR	hazard ratio
hs-CRP	high-sensitivity C-reactive protein
l_BLDL	large buoyant low-density lipoprotein
l_BLDL-C	large buoyant low-density lipoprotein-cholesterol
LDL-C	low-density lipoprotein-cholesterol
sdLDL	small dense low-density lipoprotein
sdLDL-C	small dense low-density lipoprotein-cholesterol
SNP	single-nucleotide polymorphism

sdLDL particles.¹¹ 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.¹²

The distribution of LDL subfractions is determined by both genetic and environmental factors.^{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.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^{16–18} and prospective observational studies.^{19–21} However, in most of these studies, sdLDL did not remain an independent risk predictor when adjusted for other lipid risk factor traits. 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 al²² 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 l_BLDL-C better, we investigated the association of both sdLDL-C and l_BLDL-C levels with genetic markers spanning the genome.

Materials and Methods

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

Results**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.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 1158 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 I 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 (hs-CRP) 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 A₂ 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, l_BLDL-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 l_BLDL-C quartiles, adjusted by age, race, and sex, are

Table 1. Description of 11 419 ARIC Participants at Baseline (ARIC Visit 4)

Characteristic	n=11 419
Age, y	62.83±5.67
Race	
Blacks	2515 (22)
Whites	8904 (78)
Sex	
Women	6385 (56)
Men	5034 (44)
Ever smoked cigarettes	6635 (58)
Ever drank alcohol	8989 (79)
Statin use	1308 (11.5)
Prevalent CHD	972 (8.7)
Diabetes mellitus	1943 (16.9)
Metabolic syndrome	5127 (44.6)
sdLDL-C, mg/dL	43.48±20.76
lbLDL-C, mg/dL	79.22±28.52
sdLDL-C/LDL-C	0.35±0.1496

Data are presented as mean±SD or n (%). ARIC indicates Atherosclerosis Risk in Communities; CHD, coronary heart disease; lbLDL-C, large buoyant low-density lipoprotein-cholesterol; and sdLDL-C, small dense low-density lipoprotein-cholesterol.

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.²⁴ 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 (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

Table 2. Adjusted Mean±SE or Proportions±SE of Cardiovascular Risk Factors by Quartiles of Small Dense LDL-C and P Value for Trend Across Quartiles

Characteristic	Q1	Q2	Q3	Q4	PTrend
Age, y*	62.77±0.106	62.95±0.106	62.80±0.106	62.79±0.106	0.8500
Men, %*	0.40±0.038	0.42±0.038	0.49±0.038	0.45±0.038	<0.0001
Black, %*	0.30±0.041	0.23±0.044	0.19±0.048	0.14±0.055	<0.0001
Total cholesterol, mg/dL	172.08±0.557	194.61±0.556	207.09±0.558	229.94±0.559	<0.0001
Triglycerides, mg/dL	91.70±1.370	116.20±1.368	154.69±1.373	213.49±1.374	<0.0001
LDL-C, mg/dL	97.55±0.537	119.30±0.537	129.36±0.542	145.64±0.551	<0.0001
HDL-C, mg/dL	56.16±0.271	52.37±0.271	47.85±0.272	43.47±0.272	<0.0001
BMI, kg/m ²	27.80±0.103	28.46±0.103	29.20±0.103	29.79±0.103	<0.0001
Smoking, %	59±4.0	62±4.0	57±3.9	58±4.0	0.1125
Diabetes mellitus, %	11.0±5.8	13.0±5.6	17.0±5.1	23.0±4.6	<0.0001
Hypertensive, %	40.0±4.0	46.0±3.9	51.0±3.9	54.0±3.9	<0.0001
Statin use, %	9.0±6.7	11.0±6.0	12.0±5.8	12.0±5.7	<0.0001
Metabolic syndrome, %	23.0±4.5	33.0±4.0	50.0±3.8	73.0±4.3	<0.0001
log hs-CRP	0.75±0.020	0.84±0.020	0.94±0.020	1.05±0.021	<0.0001

BMI indicates body mass index; HDL-C, high-density lipoprotein-cholesterol; hs-CRP, high-sensitivity C-reactive protein; and LDL-C, low-density lipoprotein-cholesterol. *Unadjusted mean or proportion; all others were adjusted for age, race, and sex.

Table 3. Correlation of Small Dense LDL-C With Traditional and Novel Cardiovascular Risk Factors

Risk Factor	n	Pearson <i>R</i>	<i>P</i> Value
Non-HDL-C	11 419	0.721	<0.0001
ApoB	10 720	0.706	<0.0001
Log triglycerides	11 419	0.641	<0.0001
Total cholesterol	11 419	0.608	<0.0001
LDL-C	11 234	0.543	<0.0001
Lp-PLA ₂ activity	11 108	0.319	<0.0001
HDL-C	11 419	-0.291	<0.0001
Lactate	11 417	0.253	<0.0001
Fasting plasma glucose	10 902	0.169	<0.0001
ApoA1	10 720	-0.086	<0.0001
lbLDL-C	11 234	-0.076	<0.0001
Log hs-CRP	11 202	0.072	<0.0001
hs-cTroponin T	11 130	-0.013	0.1549

Apo indicates apolipoprotein; HDL-C, high-density lipoprotein-cholesterol; hs-CRP, high-sensitivity C-reactive protein; hs-cTroponin T, high-sensitivity cardiac troponin T; lbLDL-C, large buoyant low-density lipoprotein-cholesterol; and Lp-PLA₂, lipoprotein-associated phospholipase A₂.

categories (ie, LDL-C<100 mg/dL and LDL-C≥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 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 ($P<5\times 10^{-8}$). 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). Each copy of the minor allele at this SNP raised sdLDL-C by ≈4 mg/dL and triglycerides by ≈20 mg/dL. In contrast, each copy of the minor allele lowered lbLDL-C by ≈3 mg/dL; rs508487 genotype had no significant effect on circulating LDL-C levels. Interestingly, 2 copies of the minor allele increased HDL-C by ≈5 mg/dL and total cholesterol by ≈14 mg/dL.

Given that the *PCSK7* variant (rs508487) is located in the chromosome 11 region harboring the *APOA5-APOA4-APOC3-APOA1* gene cluster, we investigated whether

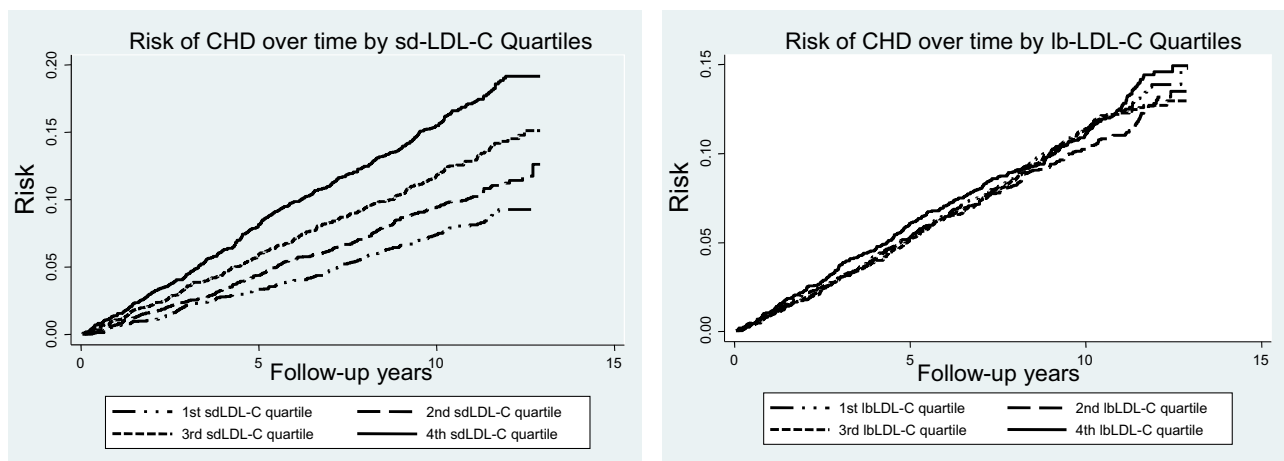


Figure 1. Cumulative incidence curves for risk of coronary heart disease (CHD) by small dense low-density lipoprotein-cholesterol (sdLDL-C) and large buoyant LDL-C (lbLDL-C) quartiles, adjusted for age, race, and sex.

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

	2	3	4	P Value*
Quartile of sdLDL-C†				
Model 1‡	1.19 (0.99–1.43)	1.44 (1.21–1.72)	2.00 (1.69–2.37)	<0.0001
Model 2§	1.10 (0.90–1.33)	1.21 (0.99–1.48)	1.51 (1.21–1.88)	0.0008
Quartile of LDL-C†				
Model 1‡	1.01 (0.85–1.21)	1.15 (0.97–1.37)	1.56 (1.32–1.83)	<0.0001
Model 2§	1.08 (0.90–1.30)	1.23 (1.03–1.47)	1.68 (1.42–1.99)	<0.0001

sdLDL-C indicates small dense low-density lipoprotein-cholesterol.

*P values ($P > \chi^2$) for linear hypothesis testing results of sdLDL-C quartiles.

†Lowest quartile (1) is reference.

‡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).

the novel association result with *PCSK7* is because of linkage disequilibrium with previously reported variants in this cluster, especially previously reported functional variants in *APOA5*. One variant, rs662799 (*APOA5* T-1131C), was in significant linkage disequilibrium with rs508487 ($R^2=0.53$). We repeated the association analysis between *PCSK7* rs508487 and sdLDL-C 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 Coronary Artery Disease Genome-Wide Replication And Meta-Analysis (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 sdLDL-C. We found a total of 7 nonsynonymous *PCSK7* variants among white ARIC participants, resulting in amino acid substitutions in the wild-type

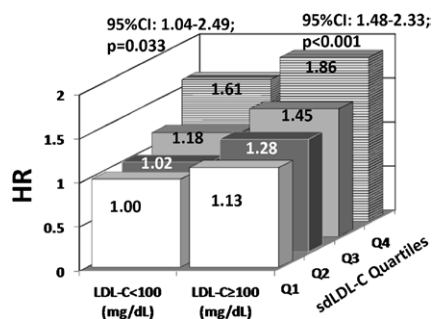


Figure 2. Adjusted hazard ratios (HRs) for incident coronary heart disease by small dense low-density lipoprotein-cholesterol (sdLDL-C) quartiles stratified by LDL-C risk categories, adjusted for age, sex, and race, smoking, body mass index, hypertension, diabetes mellitus, diabetes mellitus medications,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

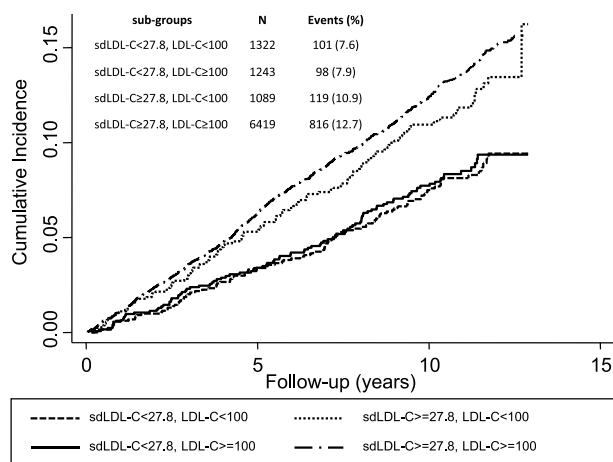


Figure 3. Cumulative incidence of cardiovascular events in sub-groups with low-density lipoprotein-cholesterol (LDL-C) <100 mg/dL (<25th percentile) and small dense LDL-C (sdLDL-C) <27.8 mg/dL (<25th percentile), from proportional hazards models adjusted for age, sex, and race.

PCSK7 protein. Because these variants had minor allele frequencies <1%, we analyzed them collectively for their effect on sdLDL-C. Individuals who were carriers of any of the rare *PCSK7* variants had a significant increase in circulating levels of sdLDL-C (≈ 7.5 mg/dL; $P=0.012$) and triglycerides ($\ln(\text{TG}) \approx 0.145$; $P=0.043$) when compared with noncarriers (Table 7).

Discussion

In the current study, we investigated the relationship between plasma levels of sdLDL-C and risk of incident CHD in the predominantly biracial ARIC study cohort using a newly developed automated homogeneous sdLDL-C assay. Elevated plasma sdLDL-C 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, sdLDL-C 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 sdLDL-C levels and other lipid traits. Subsequent examination in the CARDIoGRAM study showed a significant association of the *PCSK7* SNP rs508487 with CHD.

sdLDL-C and Risk for Incident CHD

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

Our findings related to circulating sdLDL-C levels seem in general agreement with a report from the Framingham Offspring Study, which showed that men had higher sdLDL-C levels and a higher percentage of LDL-C as sdLDL-C when compared with women.²⁵ Although we found higher mean sdLDL-C and sdLDL-C/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

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

SNP	Location	Chromosome	No. of Sign SNPs	Coded Allele	sdLDL-C					
					Allele Frequency	n	β	SE β	P Value	Gene
rs964184	11q23.3	11	12	C	0.8588	6979	-5.70008	0.511384	7.46 \times 10 ⁻²⁹	<i>APOA5/A4/C3/A1</i>
rs4420638	19q13.32	19	6	A	0.8264	6979	-5.45295	0.490271	9.77 \times 10 ⁻²⁹	<i>APOE/C1/C4/C2</i>
rs2075650	19q13.32	19	3	A	0.8611	6979	-7.15912	0.748784	1.17 \times 10 ⁻²¹	<i>TOMM40</i>
rs660240	1p13.3	1	10	C	0.7873	6979	3.45306	0.432418	1.40 \times 10 ⁻¹⁵	<i>PSRC1/CELSR2/SORT1</i>
rs6589564	11q23.3	11	18	C	0.0723	6979	5.53037	0.700672	2.95 \times 10 ⁻¹⁵	<i>BUD13</i>
rs2075290	11q23.3	11	5	C	0.0716	6979	5.48955	0.701319	4.98 \times 10 ⁻¹⁵	<i>ZNF259</i>
rs2980853	8q24.13	8	38	A	0.5315	6979	2.63962	0.353669	8.42 \times 10 ⁻¹⁴	<i>TRIB1</i>
rs1260326	2p23.3	2	3	C	0.5856	6979	-2.60009	0.366214	1.25 \times 10 ⁻¹²	<i>GCKR</i>
rs7254892	19q13.32	19	1	A	0.0534	6979	-10.9757	1.78507	7.82 \times 10 ⁻¹⁰	<i>PVRL2</i>
rs562338	2p23-2p24	2	24	A	0.1811	6979	-2.65678	0.464554	1.07 \times 10 ⁻⁰⁸	<i>APOB</i>
rs508487	11q23-q24	11	1	C	0.9402	6979	-4.95643	0.868883	1.17 \times 10 ⁻⁰⁸	<i>PCSK7</i>
rs4803760	19p13.2	19	1	C	0.7692	6979	2.73614	0.49648	3.57 \times 10 ⁻⁰⁸	<i>BCAM</i>
rs1178977	7q11.23	7	3	A	0.8000	6979	2.55226	0.466442	4.46 \times 10 ⁻⁰⁸	<i>MLXIPL</i>
rs6976930	7q11.23	7	2	A	0.2001	6979	-2.54050	0.464764	4.60 \times 10 ⁻⁰⁸	<i>BAZ1B</i>

SdLDL-C indicates small dense low-density lipoprotein-cholesterol; and SNP, single-nucleotide polymorphism.

and metabolic syndrome than the Framingham Offspring Study) and sdLDL-C assay methodologies.

Plasma levels of sdLDL-C 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.¹⁹⁻²¹ 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.^{26,27} 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 6. Association of *PCSK7* Single-Nucleotide Polymorphism rs508487 Genotype With Circulating Lipids

Lipid, mg/dL (mean \pm SE)	Genotype			P Value
	CC	CT	TT	
sdLDL-C	44.3 \pm 20.6	48.5 \pm 21.4	51.5 \pm 23.4	<0.0001
lbLDL-C	78.7 \pm 27.5	75.5 \pm 29.7	72.7 \pm 23.6	0.02
sdLDL-C/LDL-C	0.36 \pm 0.15	0.40 \pm 0.16	0.41 \pm 0.13	<0.0001
LDL-C	123.0 \pm 32.8	124.0 \pm 32.6	124.2 \pm 33.8	0.75
HDL-C	50.2 \pm 16.4	48.6 \pm 16.1	54.9 \pm 13.4	0.03
Total cholesterol	201.5 \pm 35.6	205.0 \pm 35.0	215.3 \pm 40.4	0.02
Triglycerides	141.5 \pm 67.5	162.1 \pm 77.5	181.4 \pm 88.5	<0.0001

HDL-C indicates high-density lipoprotein-cholesterol; lbLDL-C, large buoyant low-density lipoprotein-cholesterol; and sdLDL-C, small dense low-density lipoprotein-cholesterol.

Table 7. Change in Circulating Lipids Among White Carriers of Rare Nonsynonymous *PCSK7* Variants

Lipid	Change (β)	SE	P Value
sdLDL-C, mg/dL	7.53	3.00	0.012
Ln (triglycerides)	0.144	0.071	0.043
HDL-C, mg/dL	-3.58	2.12	0.091

HDL-C indicates high-density lipoprotein-cholesterol; and sdLDL-C, small dense low-density lipoprotein-cholesterol.

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 levels have been reported previously in GWAS of blood lipid levels.²⁸ Our GWAS findings are in general agreement with an earlier report by Chasman et al²⁹ 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 in women. Although the study by Chasman et al²⁹ 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.³⁰ The authors showed that the new 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 the MESA cohort. 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.^{31,32} SNP rs4420638, which is located in the *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.³³ In addition, we have previously shown an association of the SNP rs780094 in *GCKR* with metabolic syndrome prevalence and incident diabetes mellitus in the ARIC study.³⁴ rs780094 was also significantly associated with sdLDL-C ($P=4.08\times 10^{-12}$) 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,^{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 al³⁷ 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.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 *APOA5-APOA4-APOC3-APOA1*, *TRIB1*, and *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 ≈ 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.

PCSK7 encodes subtilisin-like/kexin proprotein convertase type 7 (*PCSK7*), a calcium-dependent serine endoprotease. *PCSK7* has previously been implicated as a mediator of adipogenesis³⁹ and in the processing of vascular endothelial growth factor (VEGF)-D, a critical step for binding of the angiogenic receptor VEGFR-2.⁴⁰ Furthermore, recent data show that internalization of *PCSK7* from the plasma membrane is mediated by clathrin-coated vesicles,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.^{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 *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 lbLDL-C, predicted future CHD events in ARIC participants. Furthermore, sdLDL-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 sdLDL-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 sdLDL-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 sdLDL-C with genetic variants in *PCSK7*, a member of the subtilisin-like/kexin proprotein convertase family, provides new insights into the role of this gene in lipid metabolism.

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Significance

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 subfraction 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.