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Variation in PCSK9, Low LDL Cholesterol, and Risk of Peripheral Arterial Disease

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Abstract

Background—We hypothesized that variants in *PCSK9* that lower LDL cholesterol levels are associated with reduced prevalence and incidence of peripheral artery disease (PAD).

Methods—The Atherosclerosis Risk in Communities (ARIC) Study assessed risk factors and *PCSK9* variants Y142X and C679X (relevant to blacks) and R46L (relevant to whites) in a cohort of 45–64 year olds in 1987–89 ($n=13,634$). Prevalent PAD ($n=619$ cases) was defined by an ankle-brachial index <0.9 or a history of leg claudication. Incident PAD ($n=895$) was identified from 1987 through 1998 by the same PAD criteria or a PAD hospitalization.

Results—As expected, greater LDL cholesterol was a risk factor for prevalent and incident PAD. 2.4% of blacks and 3.1% of whites were carriers of one of the race-specific *PCSK9* variants. Carriers had lower prevalence of PAD compared with non-carriers (2.3% vs. 4.6%). The corresponding age- and sex-adjusted odds ratio of PAD was 0.47 (95% confidence interval, 0.24–0.92). In contrast with the cross-sectional findings, there was no association between *PCSK9* variants and incident PAD [age- and sex-adjusted hazard ratio, 1.09 (95% confidence interval, 0.76–1.57)].

Conclusions—Our study provides mixed evidence that variation in *PCSK9* may contribute to genetic risk of PAD.

Keywords

Peripheral arterial disease; Prospective study; *PCSK9*; Total cholesterol; LDL cholesterol

1. Introduction

Peripheral arterial disease (PAD) is a major cause of morbidity in Western populations and a marker of increased risk for other cardiovascular events [1–3]. Major risk factors for PAD include age, male sex, smoking, diabetes, hypertension, dyslipidemia, and elevated fibrinogen, C-reactive protein, and other inflammatory markers [1,2].

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Cohen et al recently showed that plasma LDL cholesterol (LDL-C) concentrations and coronary heart disease (CHD) incidence are substantially reduced in individuals with certain sequence variations in the proprotein convertase subtilisin/kexin type 9 serine protease gene (*PCSK9*) [4]. In black participants in the Atherosclerosis Risk in Communities (ARIC) Study, two nonsense mutations (Y142X and C679X) were associated with a 28 percent reduction in LDL-C and an 88 percent reduction of CHD risk. In ARIC whites, a single polymorphism sequence variant (R46L) was associated with a 15 reduction of LDL-C and a 47 percent reduction of CHD risk. Others have confirmed that these loss-of-function variants are associated with reduced LDL-C [5-8]. This effect is not due to decreased LDL-C production, but rather a reduced *PCSK9* level leads to a greater LDL receptor-concentration in the liver and increased LDL-C clearance [9].

Given their association with CHD, it seems plausible that *PCSK9* variants having lifetime low LDL-C concentrations may also reduce the risk of PAD. If so, this would shed light on possible genetic determinants of PAD. We therefore examined this question in the population-based cohort of the ARIC Study.

2. Research design and methods

2.1. Population

The ARIC Study is a cohort study of cardiovascular disease in four US communities. Between 1987 and 1989, 7,082 men and 8,710 women aged 45-64 years were recruited from Forsyth County, North Carolina; Jackson, Mississippi (African Americans only); suburban Minneapolis, Minnesota; and Washington County, Maryland. There was a 46 percent response in the Jackson cohort and a 65-67 percent response in the other three cohorts. The ARIC Study protocol was approved by the institutional review board of each participating university. After written informed consent was obtained, participants underwent a baseline clinical examination (Visit 1). Follow-up examinations of the cohort occurred three times, at intervals of roughly three years. The response rates for Visits 2 (1990-1992), 3 (1993-1995), and 4 (1996-1998) were 93 percent, 86 percent, and 80 percent, respectively. Participants completed annual telephone interviews between visits and following Visit 4.

2.2. Risk factor measurements

Risk factors examined in these analyses were ascertained at Visit 1, as described in detail in the ARIC Study manuals of operation [10]. Participants were asked to fast prior to the clinical examination. Blood was drawn from an antecubital vein of seated participants into vacuum tubes containing ethylenediaminetetraacetic acid (for measurement of lipids and DNA extraction), citrate (hemostatic factors), or a serum separator gel (glucose). Serum and plasma aliquots were stored at -70°C and were shipped to central laboratories for analyses. Total cholesterol and triglycerides were measured by enzymatic methods, and high density lipoprotein cholesterol was measured after dextran-magnesium precipitation. LDL cholesterol (LDL-C) was calculated [11]. Fibrinogen, factor VIII, and von Willebrand factor were measured using published methods [12]. Serum glucose was assayed by a hexokinase/glucose-6-phosphate dehydrogenase method. Prevalent diabetes mellitus was defined as a fasting glucose ≥ 126 mg/dl [13], or a self-reported history of or treatment for diabetes. White blood cells were counted using Coulter Counters in local hospitals. Systolic (SBP) and diastolic (DBP) blood pressures were measured three times using a random-zero sphygmomanometer, and the average of the last two measurements was used for analysis. Prevalent hypertension was defined as seated DBP ≥ 90 mmHg, SBP ≥ 140 mmHg, or use of antihypertensive medications within the past two weeks. Prevalent coronary heart disease (CHD) was defined as a self-reported history of physician-diagnosed myocardial infarction (MI), coronary artery bypass surgery, coronary angioplasty, or a previous MI by electrocardiogram.

Anthropometrics were assessed with the subject wearing a scrub suit and no shoes. Body mass index was calculated (weight in kilograms/height in meters squared). Waist (umbilical level) and hips (maximum) were measured. Questionnaires assessed education, smoking status, number of cigarettes smoked per day and duration of smoking (pack years computed), usual consumption of wine, beer, and hard liquor (grams per day computed). Level of sports physical activity was assessed by the Baecke Questionnaire [14].

2.3. Genotyping

Using stored DNA from ARIC participants, fluorogenic 5'-nucleotidase assays for the *PCSK9* alleles encoding Y142X, C679X, and R46L were performed with the use of the TaqMan system (Applied Biosystems). The assays were carried out on a 7900HT Fast Real-time PCR instrument with probes and reagents purchased from Applied Biosystems. The assay failure rates for the R46L variant, the Y142X variant, and the C679X variant ranged 2.7 to 2.9 percent. The ARIC genotyping laboratory uses a 5 percent blind replicate quality-assurance program for genotype determinations; the agreement for the variants described here was 100 percent.

2.4. Ascertainment of PAD

Ankle-brachial index (ABI) was measured on nearly all participants at ARIC visit 1 (96.4%). It was later measured in random samples of examinees during visits 3 ($n = 4581$) and 4 ($n = 6419$, of whom 1124 also had a visit 3 ABI). Thus, approximately 60 percent of the original ARIC cohort had at least one follow-up ABI measurement. At visit 1, ABI was computed by dividing the average of ankle systolic BP (SBP) measurements by the average of brachial SBP measurements [14]. Using the Dinamap 1846 SX automated oscillometric device (Criticon, Tampa, FL), trained technicians measured two ankle BP, taken 5 to 8 min apart, at the posterior tibial artery in a randomly selected leg while the participant was prone. This automated BP measurement device has high validity compared with the standard Doppler ultrasound measurement and high [15]. Two brachial artery BP were measured, usually in the right arm, with the participant supine. At visits 3 and 4, the ABI was defined as the ratio of a single ankle SBP to a single brachial BP, both measured with the participant supine [16]. Between visits and through Visit 4, interviewers contacted participants annually by telephone to identify all hospitalizations and intermittent claudication symptoms (defined as exertional leg pain relieved within 10 min by resting by the Rose Questionnaire [15]). Depending on the study, the Rose Questionnaire has 9 to 92% sensitivity and 95.9 to 100% specificity in identifying PAD [17]. When a hospitalization had occurred, a trained abstractor obtained and recorded all hospital discharge diagnoses.

Prevalent PAD at the baseline visit was defined as an ABI <0.9 or intermittent claudication [18]. In individuals without prevalent PAD, PAD incidence through Visit 4 was characterized by one of the following criteria: (1) A new ABI <0.9 at either visit 3 or 4; (2) new intermittent claudication through 1998; or (3) a hospital discharge diagnosis of PAD, leg amputation, or leg revascularization procedures through 1998. All records with an International Classification of Disease, Ninth Revision code of 443.9 (claudication, peripheral arterial disease not otherwise specified, peripheral angiopathy not otherwise specified, spasm of artery), 84.11 (toe amputation), 84.12 (foot amputation), 84.15 (below-knee amputation), 84.17 (above-knee amputation), 38.18 (leg endarterectomy), 39.25 (aorto-iliac-femoral bypass), and 39.29 (leg bypass surgery) were considered to be hospitalized PAD.

2.5. Data analysis and statistical methods

From the original ARIC cohort ($n = 15,792$), we successively excluded participants who denied permission for DNA testing ($n = 137$), who were in very small racial/ethnic groups ($n = 97$), who did not provide sufficient data to determine baseline PAD status ($n = 606$), who had

missing DNA or PCSK9 genotypes ($n = 808$), or who had not fasted 8 hours ($n = 510$). This left 13,634 for baseline, cross-sectional analysis. Prevalent PAD cases ($n=619$) then were excluded, leaving 13,015 for analyses of incident PAD.

Our hypothesis was that carriers of *PCSK9* variants that lower LDL cholesterol would have reduced risk of PAD. Statistical analysis was performed by using SAS software (v. 9.1; SAS Institute, Inc., Cary, North Carolina). To explore possible confounding factors, means or prevalences of various risk factors were computed by *PCSK9* genotype and compared by t-test or chi-square test. The odds ratios (ORs) of prevalent PAD in relation to *PCSK9* variants were calculated using logistic regression. Hazard ratios (HRs) for the associations of the *PCSK9* variants with PAD incidence were calculated by using Cox proportional hazards regression. Person-years at risk were calculated from the time of baseline clinical examination until the date of PAD diagnosis, death, loss to follow-up, or December 31, 1998, whichever occurred first. The proportional hazards assumption of the Cox model was found not to be violated by testing an interaction between *PCSK9* variants and time.

3. Results

At baseline, 2.4% of blacks and 3.1% of whites in this ARIC sample of 45-64 year olds ($n = 13,634$) were carriers of the race-specific *PCSK9* variants. As expected, carriers had lower total and LDL-cholesterol (Table 1). No other potential risk factor for PAD was associated materially with *PCSK9* variation, except for a slightly higher mean pack years of smoking in whites but not blacks, and a lower prevalence of hypertension in blacks.

After pooling blacks and whites because of sample size, there were 619 prevalent PAD cases (519 with low ABI, 71 with claudication, and 29 with both). As expected, LDL-cholesterol was a risk factor for prevalent PAD: the age and sex-adjusted odds ratio of PAD per one standard deviation increment of LDL-cholesterol was 1.22 (95% confidence interval, 1.18-1.27). As shown in Table 2, carriers of the *PCSK9* variants that lower LDL-cholesterol had a lower prevalence of PAD compared with non-carriers (2.3% vs. 4.6%). The corresponding age- and sex-adjusted odds ratio of PAD was 0.47 (95% confidence interval, 0.24-0.92). When the risk factors in Table 1, other than cholesterol, were adjusted for, one at a time, there was no impact on the PAD odds ratio (range of adjusted odds ratios for *PCSK9* variant = 0.45 to 0.49). Adjustment for LDL-cholesterol, the probable primary mediator between *PCSK9* variation and PAD, attenuated the odds ratio modestly to 0.56 (95% confidence interval, 0.28-1.09).

As also shown in Table 2, among the cohort free of baseline PAD ($n = 13,015$), 895 incident PAD events occurred through 1998 (476 with new low ABI, 261 with new claudication, 158 with PAD hospitalizations). In contrast with the cross-sectional findings, there was no association between *PCSK9* variants and incident PAD. The age- and sex-adjusted hazard ratio was 1.09 (95% confidence interval, 0.76-1.57) and was unaffected by adjustment, one at a time, for non-cholesterol risk factors (hazard ratio range = 1.00 to 1.14). After adjustment for LDL-cholesterol, the hazard ratio of PAD for *PCSK9* variants (1.26, 95% confidence interval, 0.88-1.82) was still nonsignificant. LDL-cholesterol was as strongly related to incident PAD as for prevalent PAD: the age and sex-adjusted hazard ratio of PAD per standard deviation increment of LDL-cholesterol was 1.16 (95% confidence interval, 1.12-1.20).

Because the *PCSK9* variants were associated differently with prevalent PAD and incident PAD, we ran three supplemental analyses. First, we further subcategorized incident cases. The *PCSK9* hazard ratio for PAD hospitalization was 0.40 (95% confidence interval, 0.13-1.75), which is more consistent with the prevalent PAD odds ratio. In contrast, the *PCSK9* hazard ratio for new low ABI or claudication was 1.36, 95% confidence interval, 0.92-2.00. Although

neither hazard ratio was statistically significant, they were in different directions. Second, we removed from the analysis anyone without a follow-up ABI measurement, yielding 664 incident PAD cases among 8,331 subjects at risk. The age and sex-adjusted hazard ratio of 1.09 (Table 2) changed only to 1.13 (95% confidence interval, 0.74-1.73). Third, we removed anyone without a visit 4 ABI measurement, yielding 467 incident PAD cases among 5,882 at risk and a corresponding hazard ratio of 1.04 (95% confidence interval, 0.61-1.57).

4. Discussion

This population-based study provides mixed evidence that *PCSK9* variants associated with low LDL cholesterol are associated with reduced occurrence of PAD. Our cross-sectional analysis suggested a halving of PAD prevalence for carriers of these *PCSK9* variants, which is consistent with findings in this same cohort for CHD [4]. In contrast, the low cholesterol *PCSK9* variants were not associated with incident PAD. LDL cholesterol level, itself, was a comparable risk factor for PAD prevalence and incidence, so the lack of association between *PCSK9* and incident PAD is surprising.

We considered a few possible explanations for this surprise finding. First, although not statistically significant, the hazard ratios differed by the definition of incident PAD, with *PCSK9* variants inversely associated with PAD hospitalization but positively associated with new low ABI or claudication. Because our oscillometric method to assess ABI used only one leg [15], some true prevalent and incident PAD cases would have been undetected. Yet, it seems unlikely that this would create different associations of *PCSK9* with prevalent versus incident disease.

Second, a drawback of our study design was that ABI was measured at Visit 3 or 4, but not both. Some incident subclinical PAD cases would have been missed among those who did not have ABI repeated at Visit 4. Nevertheless, missing Visit 4 ABI should not be related to genotype and thus missing these ABI measures should not bias our results. Furthermore, sensitivity analyses showed that similar hazard ratios were obtained when the sample was restricted only to those with follow-up ABI.

Third, although this study was large, the *PCSK9* variants were relatively uncommon. Thus, our study had statistical power to detect only moderately large associations. The lower confidence bound for the hazard ratio for incident PAD was 0.76, so another possible explanation for our finding is that the association between *PCSK9* variants and incident PAD was inverse but we made a type 2 error.

In conclusion, our study provides mixed evidence that variation in *PCSK9* may contribute to genetic risk of PAD. There are no other studies of *PCSK9* and PAD, so replication of our results is clearly needed.

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Table 1
Race-specific, age- and sex-adjusted baseline risk factors by presence of PCSK9 variants, ARIC, 1987-89

	Blacks			Whites		
	PCSK9 ^{42X} or PCSK9 ^{679X} (n = 83)	Neither Variant (n = 3389)	p for difference	PCSK9 ^{46L} (n = 313)	Neither variant (n = 9849)	p for difference
Means						
Total cholesterol (mg/dL)	173	216	<0.0001	196	215	<0.0001
LDL-C (mg/dL)	100	139	<0.0001	118	138	<0.0001
HDL-C (mg/dL)	54.2	55.3	0.57	50.7	50.5	0.80
Triglycerides (mg/dL)	96	110	0.07	141	137	0.42
Fibrinogen (mg/dL)	313	319	0.43	293	297	0.33
Factor VIII (%)	141	146	0.28	124	126	0.31
von Willebrand Factor (%)	124	133	0.17	108	112	0.09
White Blood Cell Count (cells x 10 ⁻³ /mm ³)	5.35	5.65	0.18	6.25	6.28	0.80
Systolic BP (mm Hg)	127	128	0.46	119	118	0.41
BMI (kg/m ²)	29.3	29.5	0.82	27.0	27.0	0.88
Waist-Hip Ratio	0.91	0.92	0.78	0.93	0.93	0.55
Pack years of smoking	216	238	0.61	398	344	0.03
Ethanol intake (g/week)	18.0	32.5	0.17	40.5	45.8	0.31
Sport Activity index (range, 0-5)	2.11	2.16	0.53	2.46	2.54	0.07
Prevalences						
High school graduate (%)	57.1	59.8	0.62	83.1	82.9	0.93
Current smoking (%)	28.9	29.0	0.98	25.4	24.3	0.66
Diabetes (%)	14.1	16.2	0.61	7.8	8.7	0.59
Hypertension (%)	36.3	55.3	0.0005	27.9	27.2	0.80
Prevalent CHD (%)	3.8	3.5	0.90	6.9	5.1	0.16

Table 2Associations of *PCSK9* variants with prevalent and incident peripheral arterial disease (PAD), ARIC

Outcome	<i>PCSK9</i> Variant	
	No	Yes
Prevalent PAD (1987-89)		
N with PAD	610	9
N without PAD	12,628	387
Age- and sex-adjusted odds ratio		0.47
(95% confidence interval)		(0.24-0.92)
Age-, sex-, and LDL-C adjusted odds ratio		0.56
(95% confidence interval)		(0.28-1.09)
Incident PAD (1987-1998)		
N developing PAD	865	30
Person-years at risk	122,996	3,805
Age- and sex-adjusted hazard ratio		1.09
(95% confidence interval)		(0.76-1.57)
Age-, sex-, and LDL-C adjusted hazard ratio		1.26
(95% confidence interval)		(0.88-1.82)