ORIGINAL RESEARCH

Plasma Levels of Proprotein Convertase Subtilisin/Kexin Type 9 Are Elevated in Patients With Peripheral Artery Disease and Associated With Metabolic Disorders and Dysfunction in Circulating Progenitor Cells

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***Background*—**Proprotein convertase subtilisin/kexin type 9 (PCSK9) is involved in cholesterol homeostasis, inflammation, and oxidative stress. This study investigated the association of plasma PCSK9 levels with the presence and severity of peripheral artery disease (PAD) and with parameters of endothelial homeostasis.

***Methods and Results*—**A post hoc analysis of 2 randomized trials (115 patients, 44 with PAD and 71 without atherosclerotic disease) was conducted. Patients with PAD had significantly higher plasma PCSK9 levels than those without (471.6±29.6 versus 302.4±16.1 ng/mL, $P<0.001$). Parameters for glucose homeostasis, endothelial progenitor cell functions, apoptotic circulating endothelial cell counts, and plasma levels of vascular endothelial growth factor–A165 and oxidized low-density lipoprotein were correlated with PCSK9 concentration. By multivariable linear regression analysis, presence of PAD, plasma glucose or hemoglobin A1c levels, apoptotic circulating endothelial cell counts, and vascular endothelial growth factor–A165 concentration were found to be associated with PCSK9 levels after multivariable adjustment. Patients with extensive involvement of PAD or with severe PAD had significantly higher PCSK9 levels than those without PAD. Computed tomographic angiography showed that the numbers of chronic total occlusion sites and vessels involved were positively associated with PCSK9 levels in patients with PAD ($r=0.40$, $P=0.01$, and $r=0.36$, $P=0.02$, respectively).

***Conclusion*—**PCSK9 levels were significantly higher in patients with PAD, especially those with advanced PAD. Further large-scale studies examining the effect of PCSK9-targeting therapies or the modification of PCSK9 levels on cardiovascular outcomes in this clinical setting are warranted.

***Clinical Trial Registration*—**Cohort 1: URL: ClinicalTrials.gov. Unique identifier: NCT01952756; cohort 2: URL: ClinicalTrials.gov. Unique identifier: NCT02194686. **(*J Am Heart Assoc*. 2016;5: e003497 doi: 10.1161/JAHA.116.003497)**

Key Words: peripheral artery disease • progenitor cell • proprotein convertase subtilisin/kexin type 9

Endothelial dysfunction is involved in the initiation and potentiation of atherothrombosis.[1,2] Several cardiovascular risk factors, including metabolic disorders, negatively affect endothelial function by increasing inflammation and oxidative stress.[2–4]

Circulating endothelial progenitor cells (EPCs) play a major role in postnatal vasculogenesis for endothelial repair and neovascularization.[2,5–9] In response to endothelial injury and tissue ischemia,[10,11] EPCs proliferate and migrate to areas of injured vascular endothelium or ischemic tissue, mediated by angiogenic factors including vascular endothelial growth factor (VEGF),[12] and differentiate into mature endothelial cells in situ for endothelial repair and new blood vessel formation. EPCs are recognized as a novel biomarker of vascular endothelial function[13]; their number and functions appear to be inversely correlated with the presence of metabolic disorders[14,15] and atherosclerotic disease[16] and positively affect long-term cardiovascular outcomes.[17] Although EPCs play an important role in improving vascular disease,[2] apoptotic circulating endothelial cells (CECs) are associated with endothelial damage in atherosclerotic vascular disease.[18]

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Proprotein convertase subtilisin/kexin type 9 (PCSK9), a newly recognized protein, is involved in cholesterol homeostasis by enhancing the degradation of hepatic low-density lipoprotein (LDL) receptors.[19] Interestingly, PCSK9 is also involved in inflammatory and oxidative processes.[20–24] Plasma LDL cholesterol levels and coronary artery disease (CAD) incidence are substantially reduced in persons with certain sequence variations in the *PCSK9* gene.[25] Furthermore, plasma PCSK9 levels and cardiovascular risk factors—lipid or nonlipid—are correlated, and the associations of PCSK9 with cardiovascular health and disease render this protein worthy of attention for the prevention and treatment of atherosclerosis.[19,21,26] Moreover, plasma PCSK9 levels have been reported to be associated with the severity of CAD.[27]

Increased inflammation is associated with the prevalence of peripheral artery disease (PAD).[28,29] The circulating number and functions of EPCs are hampered in patients with PAD[16]; however, no study has investigated the association of plasma PCSK9 levels with the presence and severity of PAD. In addition, the relation of plasma PCSK9 levels to the number of apoptotic CECs, the functions of EPCs, and the levels of vasculoangiogenic biomarkers has not been evaluated previously.

In our study, we included participants from 2 double-blind, randomized, placebo-controlled trials to evaluate the association of plasma PCSK9 levels with the presence and severity of PAD and the relation of plasma PCSK9 levels to the number of apoptotic CECs, the functions of EPCs, and the levels of vasculoangiogenic biomarkers.

Methods

Patient Population

The current study included the eligible patients who were enrolled in our previous 2 prospective, double-blind, randomized, placebo-controlled trials (cohort 1, ClinicalTrials.gov identifier NCT01952756; cohort 2, ClinicalTrials.gov identifier NCT02194686) and agreed to the future use of their residual blood samples in previously obtained informed consent forms. We previously enrolled and randomized 44 participants with PAD in cohort 1 and 71 participants at high risk of cardiovascular disease (CVD) without preexisting atherosclerotic disease in cohort 2. The study of cohort 1 started in January 2012 and finished in September 2013. The study of cohort 2 started in January 2013 and finished in August 2014. The results of both studies were published, and the detailed inclusion and exclusion criteria were described previously.[2,30] In brief, patients with mild to moderate PAD and an ankle-brachial index <0.9 in one or both legs but with no obvious symptoms of intermittent claudication or critical limb ischemia were enrolled in cohort 1. In cohort 2, we enrolled eligible patients at high risk of CVD without preexisting atherosclerotic diseases such as PAD or CAD. All participants could tolerate the treatment protocol and completed the entire 3-month study without encountering cardiovascular events. This post hoc analysis did not require informed consent and was approved by the ethics committee of National Cheng Kung University Hospital (institutional review board number A-ER-104-345).

Measurement of Plasma Biomarkers

Blood samples were obtained from the peripheral veins of all patients during the run-in period. Venous blood was drawn into 50-mL EDTA-containing tubes and sent for isolation, cell culture, and assay of human EPCs. The remaining blood samples were prepared and stored for enzyme-linked immunosorbent assays, as described previously.[2,30] Plasma concentrations of biomarkers were measured using commercial kits (American Diagnostica Inc). The homeostasis model assessment index,[31] an indicator of insulin resistance, was calculated as fasting plasma insulin (in μU/mL) times fasting glucose (in mmol/L) divided by 22.5.

Determination of Circulating Numbers of EPCs and Apoptotic CECs and Isolation and Culture of EPCs

Isolation of early EPCs was performed using Ficoll density gradient centrifugation according to standard protocols, as described previously.[2,6,7,30] Colony formation by EPCs was identified and quantified, as described previously.[2,6,7,30] In brief, peripheral blood mononuclear cells (10^6 cells in each sample) were suspended in phosphate-buffered saline (Invitrogen) and incubated for 30 minutes with monoclonal antibodies against peridinin chlorophyll protein–conjugated human CD45, fluorescein isothiocyanate–conjugated human CD34, and phycoerythrin-conjugated human kinase insert domain receptor in one set. In another set, peripheral blood mononuclear cell samples were incubated with monoclonal antibodies against peridinin chlorophyll protein–conjugated human CD45 and phycoerythrin-conjugated human CD146 and then resuspended and incubated in fluorescein isothiocyanate–conjugated annexin V for 15 minutes. The cells with 10^5 events in the lymphocyte gate were acquired and analyzed using a FACSCalibur flow cytometer (BD Biosciences). EPCs were defined as cells negative for CD45 and positive for CD34 and kinase insert domain receptor, and apoptotic CECs were defined as cells negative for CD45 and positive for CD146 and annexin V.[2] All fluorescence-labeled antibodies were purchased from Becton Dickinson. Fluorescence-activated cell-sorting plots, including how the gating

was performed and how the target populations were derived from the whole cell populations, are shown in Figure 1A.

Determination of Proangiogenic Functions of EPCs

As described previously,[2,6,7,30] the migration of EPCs was measured using modified Boyden chambers. Cell proliferation and viability were analyzed using bromodeoxyuridine and XTT assays, and apoptotic cell death was detected using a terminal 2′-deoxyuridine 5′-triphosphate nick end labeling assay kit (Roche).

Dual-Energy Multislice Computed Tomographic Angiography and Processing

Dual-energy multislice computed tomographic angiography was performed on a 128-row dual-source computed tomography instrument with a dual-energy scan protocol (80 and 140 kV [peak]; Somatom Definition; Siemens AG). The injection rate of nonionic iodinated contrast material (Ultravist 370; Schering AG), the scanning protocol, and the parameters were described previously.[30] Computed tomographic angiography data were transferred to an external multimodality workplace (Syngo MMWP VA 21A; Siemens AG). The detailed data processing protocol was described previously.[30] The whole procedure was performed by an experienced radiologist who was blinded to the clinical data.

Statistical Analysis

Distributions of continuous variables in both groups were expressed as mean±SD, and median values were reported for skewed data (interquartile range). The chi-square or Fisher exact test was used for comparing categorical variables between groups. The Mann–Whitney *U* test, an unpaired Student *t* test, or a *t* test with adjustment of degrees of freedom using the Brown–Forsythe test and Welch–Satterthwaite equation (if the sample size of either group was <30 and the variances were unequal, as evaluated by Levene's test) was used for comparing continuous variables. A 1-way ANOVA with a post hoc analysis by the Games–Howell or Scheff method, as appropriate, was used for comparing plasma PCSK9 concentration categorized by ≥3 groups. A Pearson correlation was used to assess the relationship between the baseline numerical variables, including serum or plasma levels of metabolic factors and vasculoangiogenic factors, circulating EPC and apoptotic endothelial cell numbers, functions of circulating EPCs, and plasma PCSK9 levels in the entire cohort and in the patients with PAD. All single variables with a *P* value <0.1 were analyzed with a multivariable linear regression model using a stepwise regression method for the selection of covariates associated with plasma PCSK9 levels. A *P* value of <0.05 (2-sided) was considered significant. All statistical analyses were performed using SPSS for Windows (version 13.0; IBM Corp).

Results

Baseline Characteristics

For the entire study population, the mean patient age was 65.6±9.3 years, and 66.1% of the patients were male. The most prevalent cardiovascular risk factors were hypertension (76.5%) and hyperlipidemia (74.8%), followed by metabolic syndrome (60.9%) and diabetes mellitus (46.1%). The distribution of CVD in the entire study population was as follows: PAD (61.7%), CAD (26.1%), myocardial infarction (11.3%), and cerebrovascular accident (7.0%). All participants with CVD were in the PAD group. Some background characteristics and parameters were significantly different between the nonatherosclerotic disease group and the PAD group (Table 1). The PAD patients were older; had higher prevalence of diabetes mellitus, metabolic syndrome, tobacco smoking habit, and CVD; and used aspirin and statins more frequently. Significantly higher plasma levels of hemoglobin A1c and higher circulating numbers of white blood cells were found in the PAD group.

Patients with PAD had significantly higher circulating numbers of EPCs and apoptotic CECs than those without atherosclerotic disease (1.4 cells/μL [interquartile range 0.2–7.6] versus 0.2 cells/μL [interquartile range 0.1–0.6], *P*=0.001; and 0.05 cells/μL [interquartile range 0.02–0.15] versus 0.03 cells/μL [interquartile range 0.02–0.05], *P*=0.02, respectively) (Figure 1B). The in vitro proangiogenic functions of EPCs, such as colony formation and proliferation, were hampered in the patients with PAD (37.0±5.5 versus 60.1±5.5 per 1×10^6 peripheral blood mononuclear cells, *P*=0.003; 0.7±0.1 versus 1.2±0.1 per 2.5×10^5 peripheral blood mononuclear cells, *P*<0.001), whereas migration capacity was enhanced in this group (206.2±18.1 versus 107.1±9.3 cells per field, *P*<0.001) (Figure 2). Plasma levels of biomarkers, including soluble thrombomodulin, oxidized LDL, VEGF-A165, and PCSK9, were significantly higher in the PAD group (Table 2).

Association of Baseline Characteristics, Cell Biology Data, and Biomarkers and Plasma Levels of PCSK9

In the entire study population, participants with a history of diabetes mellitus, metabolic syndrome, tobacco smoking

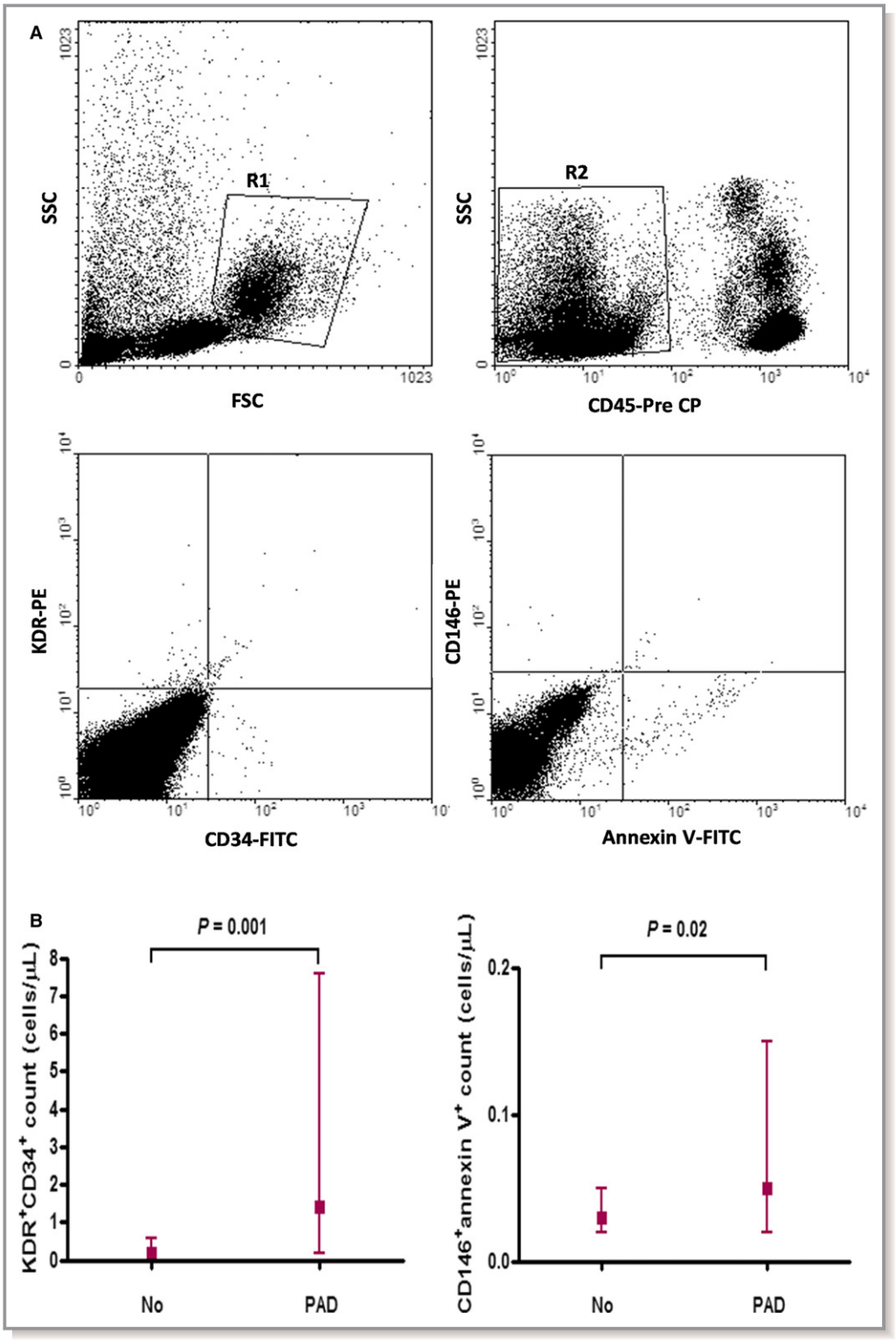

Figure 1. Flow cytometry analyses in patients with PAD or without atherosclerotic disease. A, Gating the target cell population by flow cytometry analysis. The percentages of cells that were double positive for KDR and CD34 (KDR^+CD34^+; left lower panel) or CD146 and annexin V ($CD146^+$annexin V^+; right lower panel) are shown. The surface markers were identified while the CD45-negative subpopulation was gated and adjusted for the isotype IgG control. B, Comparisons of KDR^+CD34^+ cell counts and $CD146^+$annexin V^+ cell counts between both groups. *P* values were calculated by Mann–Whitney *U* test. FITC indicates fluorescein isothiocyanate; FSC, forward scatter; KDR, kinase insert domain receptor; No, no preexisting atherosclerotic disease; PAD, peripheral artery disease; PCSK9, proprotein convertase subtilisin/kexin type 9; PE, phycoerythrin; Pre CP, peridinin chlorophyll protein; R, region; SSC, side scatter.

Table 1. Baseline Characteristics in the Overall Cohort and Comparisons of These Parameters Between Patients in the Nonatherosclerotic Disease and PAD Groups

	Overall Cohort (n=115)	Nonatherosclerotic Disease (n=71)	PAD (n=44)	*P* Value
Age, y	65.6±9.3	62.2±7.7	71.1±9.2	<0.001
Male sex	76 (66.1)	46 (64.8)	30 (68.2)	0.71
Underlying disease				
Diabetes mellitus	53 (46.1)	26 (36.2)	27 (61.4)	0.01
Hypertension	88 (76.5)	56 (78.9)	32 (72.7)	0.45
Hyperlipidemia	86 (74.8)	56 (78.9)	30 (68.2)	0.20
Metabolic syndrome	70 (60.9)	33 (46.5)	37 (84.1)	<0.001
Tobacco smoking	23 (20.0)	10 (14.1)	13 (29.5)	0.04
Chronic kidney disease	13 (11.3)	9 (12.7)	4 (9.1)	0.76
Coronary artery disease	30 (26.1)	0	30 (68.2)	<0.001
Myocardial infarction	13 (11.3)	0	13 (29.5)	<0.001
Cerebrovascular accident	8 (7.0)	0	8 (18.2)	<0.001
Aspirin use	60 (52.2)	25 (35.2)	35 (79.5)	<0.001
Clopidogrel use	8 (7.0)	5 (7.0)	3 (6.8)	1.00
ACEI use	20 (17.4)	13 (18.3)	7 (15.9)	0.74
ARB use	51 (44.3)	30 (42.3)	21 (47.7)	0.57
CCB use	62 (53.9)	37 (52.1)	25 (56.8)	0.62
Diuretic use	28 (24.3)	16 (22.5)	12 (27.3)	0.57
Statin use	56 (48.7)	28 (39.4)	28 (63.6)	0.01
Thiazolidinedione use	12 (10.4)	5 (7.0)	7 (15.9)	0.21
Fasting plasma glucose, mg/dL	119.9±4.7	114.5±3.9	128.5±10.6	0.22
Hemoglobin A1c, %	6.6±0.1	6.4±0.1	7.0±0.3	0.05
Fasting insulin, mU/L	9.7 (6.1–16.0)	9.8 (6.7–16.0)	9.6 (5.1–17.1)	0.79
HOMA index, median	2.5 (1.5–5.1)	2.5 (1.6–5.0)	2.5 (1.3–5.2)	0.98
Body weight, kg	72.5±1.3	73.7±1.7	70.5±1.8	0.23
Waist circumference, cm	97.0±1.0	95.7±1.3	99.1±1.4	0.10
Body mass index, kg/m^2	27.9±0.4	28.4±0.5	27.1±0.5	0.11
Blood pressure, mm Hg				
Systolic	133.9±1.6	133.1±1.8	135.2±3.0	0.54
Diastolic	78.6±1.3	79.1±1.3	77.9±2.6	0.66
Heart rate, beats/min	77.8±1.2	76.6±1.4	79.6±2.2	0.25
White blood cell count, $10^3/\mu L$	6553.9±167.9	6239.4±204.3	7061.4±275.6	0.02
Hemoglobin, g/dL	14.0±0.3	14.2±0.4	13.7±0.3	0.33
Platelet count, $10^3/\mu L$	203.7±4.1	209.2±4.9	194.8±7.3	0.09
Total cholesterol, mg/dL	180 (157–200)	185 (164–202)	172 (147–193)	0.07
Triglyceride, mg/dL	147.9±9.8	134.2±8.9	170.0 (20.8)	0.12
HDL cholesterol, mg/dL	51.4±1.2	52.7±1.5	49.3±1.9	0.16
LDL cholesterol, mg/dL	114.4±3.1	121.3±4.2	103.3±3.9	0.002

Data are expressed as mean±SD, n (%), or median (interquartile range), as appropriate. *P* values comparing the nonatherosclerotic disease and PAD groups were obtained using an unpaired Student *t* test, Mann–Whitney *U* test, or chi-square test. ACEI indicates angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; CCB, calcium channel blocker; HDL, high-density lipoprotein; HOMA, homeostasis model assessment; LDL, low-density lipoprotein; PAD, peripheral artery disease.

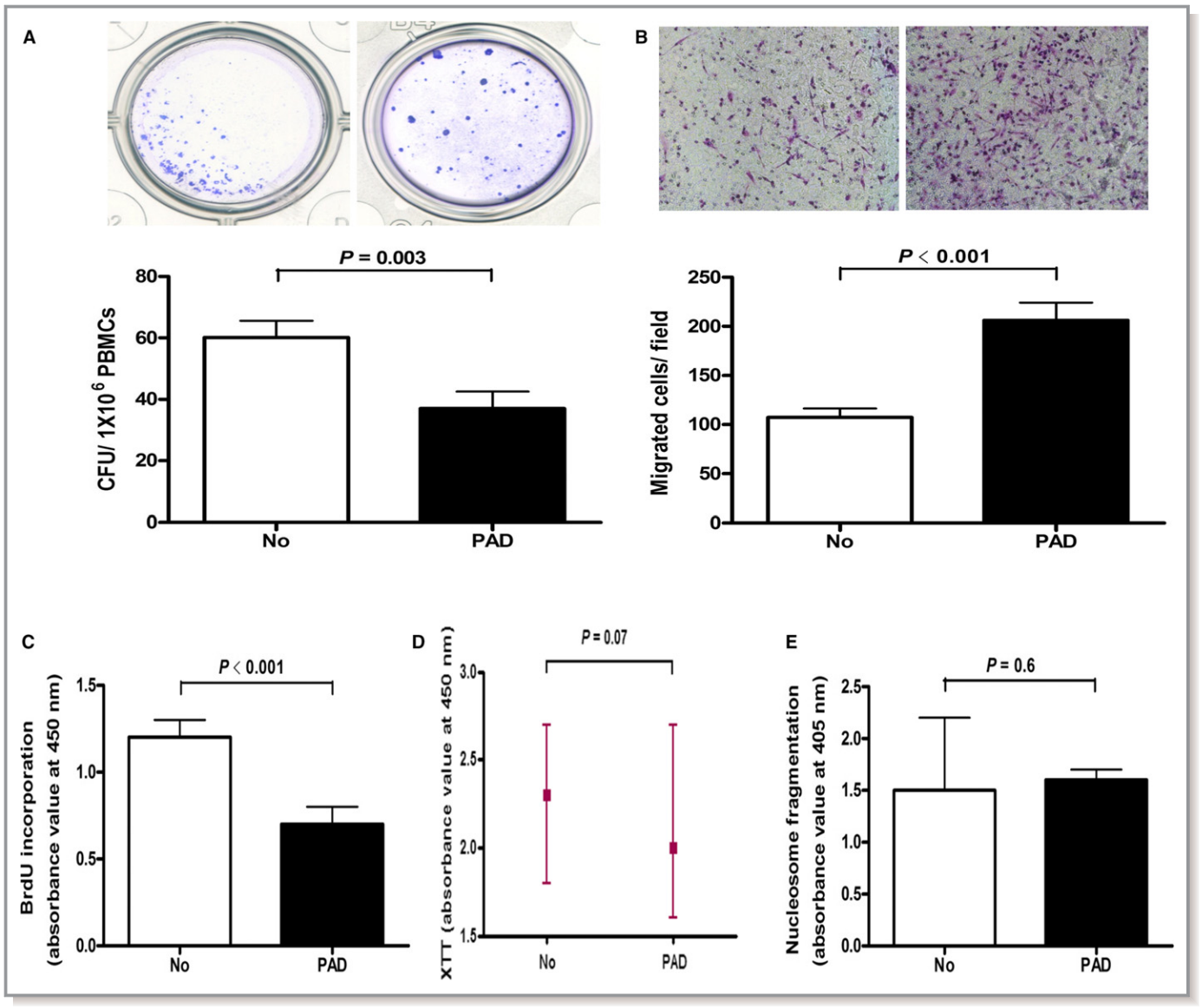

Figure 2. Cell biological studies in patients with PAD or without atherosclerotic disease. Representative photographs and quantitative analyses of colony-forming units (A) and migrated cells (B). Cells and colonies were fixed with methanol and stained with Giemsa reagent and appeared in purple. Original magnification was ×100 (B). Comparisons of BrdU incorporation (C), XTT (D), and nucleosome fragmentation of EPCs (E) between patients in the nonatherosclerotic disease and PAD groups were shown. *P* values were calculated by Mann–Whitney *U* test in panel D and by unpaired Student *t* test in other panels. BrdU indicates bromodeoxyuridine; CFU, colony-forming unit; EPC, endothelial progenitor cell; No, no preexisting atherosclerotic disease; PAD, peripheral artery disease; PBMC, peripheral blood mononuclear cell.

habit, PAD, CAD, or statin use had significantly higher plasma PCSK9 levels than those without such history (Table 3). Moreover, some metabolic factors, plasma biomarkers, the number and functions of EPCs, and the number of apoptotic CECs were significantly correlated with plasma PCSK9 levels (Table 4). Levels of fasting plasma glucose, hemoglobin A1c, homeostasis model assessment index, VEGF-A165, oxidized LDL, numbers of circulating EPCs and apoptotic endothelial cells, and in vitro measurements of EPC migration were positively correlated with plasma PCSK9 levels, whereas colony-forming units and bromodeoxyuridine incorporation potential were inversely associated with plasma PCSK9 levels.

PAD Is Associated With Plasma Levels of PCSK9 After Adjustment for Other Variables

PAD was highly correlated with CAD, whereas fasting plasma glucose levels were highly correlated with hemoglobin A1c levels. Consequently, as we evaluated the covariates associated with PCSK9 levels, we performed multivariable linear regression analyses by considering either PAD or CAD and

Table 2. Baseline Plasma Concentrations of Biomarkers in the Overall Cohort and Comparisons of These Biomarkers Between Patients in the Nonatherosclerotic Disease and PAD Groups

	Overall Cohort (n=115)	Nonatherosclerotic Disease (n=71)	PAD (n=44)	*P* Value
hsCRP, mg/L	1.5 (0.7–2.7)	1.4 (0.9–2.7)	1.6 (0.5–3.2)	0.71
Oxidized LDL, U/L	54.6±19.5	50.4±20	61.4±16.9	0.003
Soluble TM, pg/mL	5723.7±495.8	4253.3±127.0	8029.4±1183.9	0.003
VEGF-A165, pg/mL	394.1±30.0	297.3±31.1	545.8±52.3	<0.001
SDF-1α, pg/mL	2029.1±106.8	1914.6±148.4	2208.6±143.2	0.18
Adiponectin, ng/mL	5242.2±392.7	5739.2±414.3	4462.9±763.4	0.11
PCSK9, ng/mL	367.1±16.9	302.4±16.1	471.6±29.6	<0.001

Data are expressed as mean±SD or median (interquartile range), as appropriate. *P* values comparing the nonatherosclerotic disease and PAD groups were obtained using an unpaired Student *t* test or Mann–Whitney *U* test. hsCRP indicates high sensitivity C-reactive protein; LDL, low-density lipoprotein; PAD, peripheral artery disease; PCSK9, proprotein convertase subtilisin/kexin type 9; SDF-1α, stromal cell–derived factor 1α; TM, thrombomodulin; VEGF, vascular endothelial growth factor.

Table 3. Association of Baseline Characteristics and Plasma Levels of PCSK9 in the Overall Cohort

	PCSK9 Levels, ng/mL		
	Patients With History of Variable	Patients Without History of Variable	*P* Value
Male sex	471.6±29.6	302.4±16.1	0.84
Underlying disease			
Diabetes mellitus	423.8±24.0	318.7±22.0	0.002
Hypertension	357.7±18.7	397.7±37.9	0.32
Hyperlipidemia	363.9±19.3	376.6±35.1	0.75
Metabolic syndrome	408.8±22.6	302.2±21.9	0.001
Tobacco smoking	437.4±38.9	349.6±18.4	0.04
Peripheral artery disease	471.6±29.6	302.4±16.1	<0.001
Coronary artery disease	447.4±33.7	338.8±18.6	0.004
Myocardial infarction	404.1±41.0	362.4±18.3	0.44
Cerebrovascular accident	396.3±59.8	364.9±17.6	0.64
Chronic kidney disease	323.0±55.3	372.7±17.7	0.35
Aspirin use	376.2±23.9	357.2±23.9	0.58
Clopidogrel use	344.3±51.5	368.8±17.8	0.71
RASI use	367.5±21.6	366.5±27.4	0.98
CCB use	378.6±22.2	353.7±25.8	0.47
Diuretic use	380.6±40.3	362.8±18.3	0.65
Statin use	429.7±26.2	307.7±18.6	<0.001
Thiazolidinedione use	373.2±50.8	366.4±18.0	0.90

Data are expressed as mean±SD. *P* values comparing groups were obtained using an unpaired Student *t* test or a *t* test with adjustment of the degrees of freedom using the Brown–Forsythe test and Welch–Satterthwaite equation if the sample size of either group was <30 and the variances were unequal, as evaluated by Levene's test. CCB indicates calcium channel blocker; PCSK9, proprotein convertase subtilisin/kexin type 9; RASI, renin–angiotensin system inhibitor.

either fasting plasma glucose levels or hemoglobin A1c levels in different models. Our data showed that in the presence of PAD, baseline plasma glucose or hemoglobin A1c levels, circulating CD146- and annexin V–positive counts, and VEGF-A165 concentration were significantly associated with PCSK9 levels after multivariable adjustment (Table 5).

During examination of the association of the baseline characteristics with PCSK9 levels in the PAD and

Table 4. Significant Correlation Between Baseline Numerical Variables and Plasma Levels of PCSK9 in the Overall Cohort

	PCSK9 Level	
	r	*P* Value
Fasting plasma glucose, mg/dL	0.32	0.001
Hemoglobin A1c, %	0.32	0.001
HOMA index	0.19	0.05
Colony-forming units per 1×10^6 PBMCs	−0.19	0.04
KDR^+CD34^+ count, cells/μL	0.22	0.02
$CD146^+$annexin V^+ count, cells/μL	0.22	0.02
BrdU incorporation, absorbance value at 450 nm	−0.31	0.001
Migrated cells per field	0.19	0.05
VEGF-A165, pg/mL	0.31	0.001
Age, yr	0.16	0.09
LDL cholesterol, mg/dL	−0.16	0.08
Oxidized LDL, U/L	0.20	0.03

BrdU indicates bromodeoxyuridine; HOMA, homeostasis model assessment; KDR, kinase insert domain receptor; LDL, low-density lipoprotein; PBMC, peripheral blood mononuclear cell; PCSK9, proprotein convertase subtilisin/kexin type 9; VEGF, vascular endothelial growth factor.

Table 5. Associated Covariates of Plasma Levels of PCSK9 Evaluated by Linear Regression Analysis

	Model 1		Model 2		Model 3		Model 4	
	β	*P* Value	β	*P* Value	β	*P* Value	β	*P* Value
Peripheral artery disease	0.41	<0.001	0.39	<0.001	—	—	—	—
Fasting plasma glucose, mg/dL	0.27	0.002	—	—	0.30	0.001	—	—
Hemoglobin A1c (%)	—	—	0.24	0.01	—	—	0.31	0.001
$CD146^{+}$annexin V^{+} count, cells/μL	—	NS	—	NS	0.19	0.03	0.21	0.02
VEGF-A165, pg/mL	—	NS	—	NS	0.25	0.01	0.23	0.01

Model 1: peripheral artery disease was adjusted for diabetes mellitus, metabolic syndrome, smoking, statin use, age, fasting plasma glucose level, low-density lipoprotein cholesterol level, oxidized low-density lipoprotein level, colony-forming units, double-positive kinase insert domain receptor and CD34 counts, double-positive CD146 and annexin V counts, bromodeoxyuridine incorporation, migrated cells, and VEGF-A165 level. Model 2 included all variables in model 1 except that fasting plasma glucose level was replaced with hemoglobin A1c. In model 3, coronary artery disease was adjusted for all variables in model 1 except peripheral artery disease. Model 4 included all variables in model 3 except that fasting plasma glucose level was replaced with hemoglobin A1c. The — symbol indicates no data. NS indicates nonsignificant; PCSK9, proprotein convertase subtilisin/kexin type 9; VEGF, vascular endothelial growth factor.

nonatherosclerotic disease groups (Table 6), we discovered that patients with histories of diabetes mellitus and metabolic syndrome had significantly higher PCSK9 levels in the nonatherosclerotic disease group, whereas patients with history of statin use had significantly higher PCSK9 levels in the PAD group. Plasma PCSK9 levels did not significantly

Table 6. Association of Baseline Characteristics and Plasma Levels of PCSK9 in Patients With PAD and Nonatherosclerotic Disease

	PCSK9 Levels, ng/mL					
	Nonatherosclerotic Disease			PAD		
	Patients With History of Variable	Patients Without History of Variable	*P* Value	Patients With History of Variable	Patients Without History of Variable	*P* Value
Male sex	292.5±19.5; n=46	320.6±28.6; n=25	0.41	487.8±35.0; n=30	436.9±55.7; n=14	0.43
Underlying disease						
Diabetes mellitus	370.8±28.9; n=26	262.8±16.8; n=45	0.001	474.7±35.9; n=27	466.6±52.6; n=17	0.90
Hypertension	302.4±18.5; n=56	302.1±33.4; n=15	0.99	454.5±34.2; n=32	517.1±59.3; n=12	0.35
Hyperlipidemia	300.0±19.0; n=56	311.3±28.9; n=15	0.78	483.2±33.1; n=30	446.6±61.6; n=14	0.57
Metabolic syndrome	341.2±28.5; n=33	268.7±15.6; n=38	0.03	469.2±31.5; n=37	484.3±89.2; n=17	0.85
Tobacco smoking	355.4±45.8; n=10	293.7±17.1; n=61	0.19	500.4±54.2; n=13	459.5±35.7; n=31	0.54
PAD						
Coronary artery disease	—	—	—	447.4±33.7; n=30	423.4±58.1; n=14	0.24
Myocardial infarction	—	—	—	404.1±41.0; n=13	499.9±37.5; n=31	0.14
Cerebrovascular accident	—	—	—	396.3±59.8;±n=8;	488.3±33.3; (n=36;	0.24
Chronic kidney disease	270.9±33.6; n=9	306.9±17.8; n=62	0.46	440.2±162.4; n=4	474.7±29.3; n=40	0.85
Aspirin use	274.6±23.2; n=25	317.5±21.3; n=46	0.21	448.8±32.5; n=35	560.1±65.6; n=9	0.13
Clopidogrel use	250.5±28.0; n=5	306.3±17.2; n=66	0.38	500.5±51.2; n=3	469.5±31.6; n=41	0.64
RASI use	299.2±20.3; n=42	307.1±26.8; n=29	0.82	470.0±37.2; n=28	474.3±50.4; n=16	0.95
CCB use	319.2±20.8; n=37	284.1±24.9; n=34	0.28	466.5±40.3; n=25	478.3±44.7; n=19	0.85
Diuretic use	296.5±38.8; n=16	304.1±17.7; n=55	0.85	492.7±67.6; n=12	463.7±32.5; n=32	0.67
Statin use	322.0±31.0; n=28	289.6±17.5; n=43	0.33	537.3±31.3; n=28	356.5±49.2; n=16	0.002
Thiazolidinedione use	298.6±17.8; n=5	302.7±17.3; n=66	0.95	426.4±82.6; n=7	480.1±31.9; n=37	0.51

Data are expressed as mean±SD. *P* values comparing groups were obtained using an unpaired Student *t* test or a *t* test with adjustment of degrees of freedom using the Brown–Forsythe test and Welch–Satterthwaite equation if the sample size of either group was <30 and the variances were unequal, as evaluated by Levene's test. The — symbol indicates no data. CCB indicates calcium channel blocker; PAD, peripheral artery disease; PCSK9, proprotein convertase subtilisin/kexin type 9; RASI, renin–angiotensin system inhibitor.

Table 7. Association of the Severity of PAD and Plasma Levels of PCSK9 in Patients with Peripheral Artery Disease

	PCSK9 Levels, ng/mL			
	Patients With Presentation of Variable	Patients Without Presentation of Variable	*r*	*P* Value
Two limbs involved diagnosed by ABI	548.0±43.6; n=17	423.5±37.3; n=27		0.04
Severe peripheral artery disease diagnosed by ABI	576.5±59.4; n=10	440.7±32.7; n=34		0.05
Two limbs involved diagnosed by CTA	466.1±35.7; n=31	484.7±54.8; n=13		0.78
CTO diagnosed by CTA	476.4±34.3; n=36	450.1±54.6; n=8		0.74
Number of CTOs assessed by CTA			0.40	0.01
Number of vessels involved assessed by CTA			0.36	0.02

Data are expressed as mean±SD. *P* values comparing the nonatherosclerotic disease and PAD groups were obtained using an unpaired Student *t* test or Mann–Whitney *U* test.. ABI indicates ankle-brachial index; CTA, computed tomographic angiography; CTO, chronic total occlusion; PAD, peripheral artery disease; PCSK9, proprotein convertase subtilisin/kexin type 9.

differ according to participant sex or ethnic background among either PAD patients or those without atherosclerotic disease.

Plasma PCSK9 Levels Are Correlated With the Severity and Extent of PAD

Patients with extensive involvement of PAD (ankle-brachial index <0.9 in both lower limbs) and severe PAD (ankle-brachial index <0.6 in at least 1 lower limb) had significantly higher PCSK9 levels than those without such involvement (548.0±43.6 versus 423.5±37.3 ng/mL, *P*=0.04; and 576.5±59.4 versus 440.7±32.7 ng/mL, *P*=0.05, respectively) (Table 7 and Figure 3A and 3B). In counting the number of vessels involved as assessed by computed tomographic angiography, we defined significant stenosis as >70% luminal narrowing in diameter compared with the adjacent vessel size. We grouped lower limb vessels into 5 groups in 1 limb: iliac, femoropopliteal, anterior tibial/dorsal pedis, posterior tibial/medial plantar, and peroneal arteries. We found that the number of chronic total occlusion sites and the number of vessels involved, as assessed by computed tomographic angiography, were positively associated with PCSK9 levels in patients with PAD (*r*=0.40, *P*=0.01; and *r*=0.36, *P*=0.02, respectively). Furthermore, we reclassified PAD lesions according to an updated recommendation of the Inter-Society Consensus for the Management of Peripheral Artery Disease (TASC II).[32] The most severe lesion in each

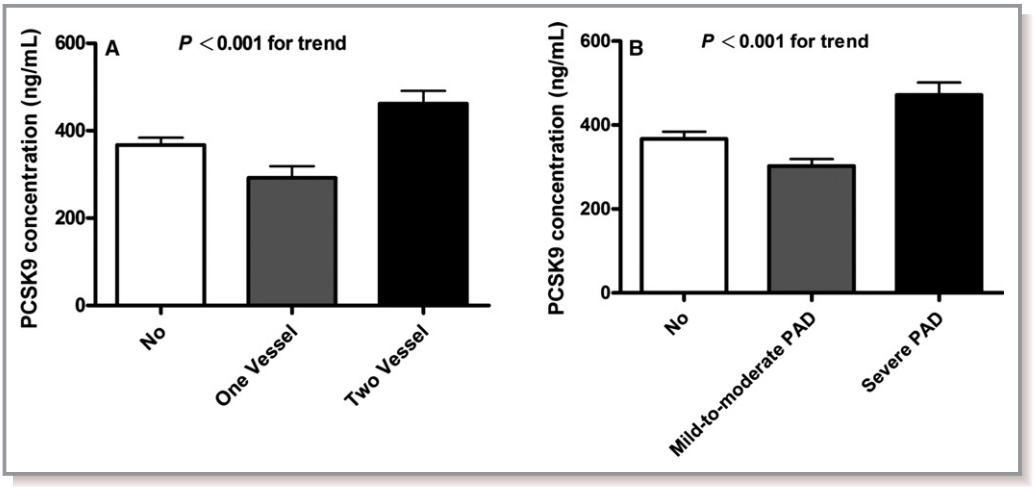

Figure 3. PCSK9 levels were compared with the extent (A) and severity (B) of PAD. Severe PAD was defined as having an ankle-brachial index <0.6, and mild to moderate PAD was defined as having an ankle-brachial index between 0.6 and 0.9. *P* values for the trends in panels A and B were calculated by 1-way ANOVA. No indicates no preexisting atherosclerotic disease; PAD, peripheral artery disease; PCSK9, proprotein convertase subtilisin/kexin type 9.

group of lower limb vessels (as defined above) was selected and stratified into a TASC II category. We found that the reclassification of PAD lesions according to the TASC II classification was not associated with plasma PCSK9 levels in patients with PAD (ANOVA, P=0.28).

Discussion

In the current study, we conducted a post hoc analysis of 2 prospective, randomized, double-blind, placebo-controlled trials and found that PCSK9 levels were significantly higher in patients with PAD, especially those with extensive, severe, and complicated PAD. A history of PAD was associated with higher PCSK9 levels after adjustment for a number of covariates including history of CAD, metabolic disorders, and statin use. Circulating EPC dysfunction, in particular, the number of apoptotic CECs, and some vasculoangiogenic and oxidative biomarkers such as VEGF-A165 were significantly correlated with PCSK9 levels.

To date, no study has investigated PCSK9 levels in patients with PAD. In addition to lipid-related effects on atherosclerosis, PCSK9 also has an off-target proatherogenic effect[19] because it is also expressed in atherosclerotic plaques.[21,33] The vascular origin of PCSK9 secreted by vascular smooth muscle cells may modulate the cellular composition of atherosclerotic plaques by directly reducing LDL receptor expression and LDL cholesterol uptake of macrophages, resulting in vascular lipid accumulation and oxidation.[19,21,33] Previous studies have shown that some demographic and metabolic factors, such as age, glucose, obesity or body mass index, and blood pressure are positively correlated with PCSK9 concentration.[19,34,35] PCSK9 has been shown to be associated with inflammation[20,24] because plasma PCSK9 levels are correlated with fibrinogen levels,[22] white blood cell count,[23] the severity of coronary stenosis,[27] and carotid intima–media thickness in CAD patients.[36] PAD shares common pathogenesis mechanisms of atherosclerosis with CAD,[37] and patients with PAD have a higher prevalence of concomitant CAD.[38] Taken together, the concentration of PCSK9 in plasma is presumed to be higher in patients with PAD. The current study confirms our hypothesis that the concentration of PCSK9 in plasma is significantly higher in patients with PAD than in patients at high risk of CVD but without atherosclerotic disease, an effect that is independent of the presence of CAD, metabolic disorders, or statin use. The association of PCSK9 concentration with the extent and severity of stenosis in peripheral limbs further highlights the role of PCSK9 in modulation of the development and progression of atherosclerosis.

The relation of EPC functions to PCSK9 concentration has not been reported previously. Only a few experimental studies have demonstrated the association of PCSK9 with cellular functions.[39–44] Seidah and Prat,[39] for example, suggested that PCSK9 may play a critical role in various functions including the growth and differentiation of progenitor cells. Some reports revealed that PCSK9 expression is positively associated with apoptosis in vascular endothelial cells, tumor cells, and neurons.[40–42] The proprotein convertase family is involved in tumor cell proliferation.[43,44] Our clinical study confirms that the circulating number of apoptotic CECs was positively correlated with PCSK9 concentration, and a number of EPC functions were inversely associated with PCSK9 concentration, suggesting a detrimental effect of this protein on endothelial repair and vasculogenesis. Our study, however, also showed that the circulating number of EPCs and in vitro migration might be positively correlated with PCSK9 concentration, even though the effects were attenuated after adjustment. Previous studies found that the circulating number and functions of EPCs may not always be parallel.[2,30,45] Moreover, the induction of increased levels of dysfunctional high-density lipoprotein by a cholesteryl ester transfer protein inhibitor was reported previously.[46] We speculate that PCSK9 might increase the number of dysfunctional EPCs, although the possibility of a double-edged effect of PCSK9 on endothelial repair and vasculogenesis could not be excluded.

Proprotein convertases have been found to be candidate VEGF convertases that can process pro-VEGF,[43,44] implying that PCSK9 and VEGF levels should be positively correlated. VEGF is effective in stimulating the mobilization and migration of bone marrow–derived EPCs by activating the Akt signaling pathway.[8] Accordingly, in the current study, the associations of the circulating number of EPCs and in vitro migration with PCSK9 concentration might be related to the positive effect of PCSK9 on VEGF.

Previous experimental studies linked PCSK9 with oxidative stress. Reactive oxygen species upregulate PCSK9 expression,[24,47] whereas PCSK9, in turn, positively influences the expression of lectin-like oxidized LDL receptor 1.[24] Furthermore, both oxidative stress and PCSK9 play important roles in inducing inflammation. Inhibition of PCSK9 suppresses the inflammatory response induced by oxidized LDL in macrophages.[48] Our clinical data revealed that oxidized LDL levels were positively correlated with PCSK9 concentration.

Our data, congruent with a previous study,[35] showed that some metabolic parameters, such as fasting plasma glucose levels, are correlated with PCSK9 concentration; however, our study did not demonstrate significant correlations between PCSK9 concentration and some demographic or metabolic parameters, such as age, blood pressure, body mass index, and lipid profile. This discrepancy might be caused by use of a different study population compared with

previous studies that enrolled participants from the general population or children and adolescents in communities.[34,35] In focusing on patients with CAD and including the multivariable adjustment, many demographic and metabolic parameters were no longer significantly and independently correlated.[27]

Statin affects PCSK9 concentration by upregulating the transcription factor sterol regulatory element–binding protein 2.[49] Despite not excluding participants with baseline statin treatment in the current study because of the high prevalence of statin use in patients with PAD or at high risk of CVD, the presence of PAD, fasting glucose levels or hemoglobin A1c, circulating numbers of apoptotic CECs, and VEGF levels were still significantly associated with PCSK9 concentration after adjusting for statin use.

Our data, in agreement with a previous study,[50] showed that PCSK9 concentrations were similar in patients with history of acute or nonacute myocardial infarction. A previous experimental study demonstrated that, in parallel with an elevation in the plasma PCSK9 concentration, hepatic PCSK9 expression was transiently upregulated in the acute stage of myocardial infarction in rats.[51] The previous clinical trial further confirmed that plasma PCSK9 levels were elevated either immediately prior to or during myocardial infarction.[50]

This study was limited by a moderate sample size, leading to a decrease in statistical power. The nature of a post hoc analysis may not exclude the possibility of selection bias.

Conclusions

Owing to a major role of EPCs and VEGF in the endothelial repair and neovascularization mechanism, PCSK9 may have a prognostic impact for patients who have ischemic disease or who are at high risk of CVD. A recent study[52] demonstrated that circulating PCSK9 levels could predict the future risk of cardiovascular events independently of established CVD risk factors. Further large-scale studies examining the effect of PCSK9-targeting therapies or the modification of PCSK9 levels on cardiovascular outcomes in patients who have PAD or who are at high risk of CVD are warranted.

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Disclosures

None.

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