ORIGINAL RESEARCH

PCSK9 Protein and rs562556 Polymorphism Are Associated With Arterial Plaques in Healthy Middle-Aged Population: The STANISLAS Cohort

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Background—PCSK9 (Proprotein convertase subtilisin/kexin type 9) binds low-density lipoprotein receptor, preventing its recycling. PCSK9 is a risk predictor and a biotarget in atherosclerosis. The PCSK9-rs562556 variant has been reported as a gain-of-function mutation. The aim of this study was to determine whether the PCSK9–low-density lipoprotein receptor–rs562556 axis is associated with carotid artery plaques between 2 visits separated by almost 20 years in a longitudinal population cohort.

Methods and Results—The STANISLAS (Suivi Temporaire Annuel Non-Invasif de la Santé des Lorrains Assurés Sociaux) cohort is a longitudinal familial cohort from the Lorraine region of France. Participants attending 2 visits (visit 1 and visit 4) separated by 18.5 years (mean) were included (n=997). Carotid artery plaques were determined with standardized vascular echography. The mean age of the adult population at visit 1 was 42±5 years. At visit 4, 203 (20.4%) participants had arterial plaques. Participants who developed arterial plaques were older (42.7±5.4 versus 41.7±4.7 years), more often male (60% versus 49%), smokers (29% versus 18%), with diabetes mellitus (6% versus 3%), and higher cholesterol levels (low-density lipoprotein cholesterol, 1.6±0.4 versus 1.5±0.3 g/L) (all $P<0.05$). The independent factors associated with arterial plaques were age, smoking, and low-density lipoprotein cholesterol. Higher PCSK9 levels were associated with arterial plaques on top of the clinical model (odds ratio, 2.14; 95% CI,= 1.28–3.58); the missense mutation coding the single-nucleotide polymorphism rs562556 was associated with both higher PCSK9 concentration and incident carotid arterial plaques.

Conclusions—Higher PCSK9 concentration was associated with the development of arterial plaques almost 20 years in advance in a healthy middle-aged population. Mutations of the single-nucleotide polymorphism rs562556 associated with both PCSK9 levels and arterial plaques reinforce the potential causality of our findings. PCSK9 inhibitors could be useful for primary cardiovascular prevention. **(*J Am Heart Assoc*. 2020;9:e014758. DOI: 10.1161/JAHA.119.014758.)**

Key Words: STANISLAS cohort • arterial plaques • PCSK9 • rs562556 mutations • LDL receptor • cholesterol

Atherosclerotic cardiovascular disease is the leading cause of death worldwide.[1] For nearly 2 decades, several guidelines or consensus statements have highlighted that the detection of carotid plaque is an important clinical predictor of future adverse cardiovascular events.[2,3] Among 13 145 participants in the ARIC (Atherosclerosis Risk in Communities) study, free of cardiovascular disease at the beginning of the follow-up and followed for a mean of 15.1 years (accumulating a total of 1812 major cardiovascular events), the presence of carotid plaques was independently associated with incident

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Clinical Perspective

What Is New?

- PCSK9 (Proprotein convertase subtilisin/kexin type 9) binds low-density lipoprotein receptor, preventing its recycling; thus, PCSK9 is a risk predictor and a biotarget in atherosclerosis.
- The PCSK9-rs562556 variant has been reported as a gain-of-function mutation.
- In this longitudinal general-population study, higher PCSK9 levels were independently associated with incident arterial plaques on top of a well-calibrated clinical model; the missense mutation coding the single-nucleotide polymorphism rs562556 was associated with both higher PCSK9 concentration and incident carotid arterial plaques, reinforcing the potential causality of our findings.

What Are the Clinical Implications?

- PCSK9 inhibitors could be useful for primary cardiovascular prevention.

major cardiovascular events and improved the predictive capacity of the clinical model using "classic" risk factors, such as age, smoking, and cholesterol.[4] Whether a strategy aimed at modifying patients' risk factors based on the presence of carotid plaques would be effective in reducing cardiovascular risk is yet to be proven.[5]

Low-density lipoprotein cholesterol (LDL-C) is a strong, independent, and modifiable risk factor for developing cardiovascular disease.[6] Lowering LDL-C, mainly with statins, has decreased the risk of cardiovascular events over the past decades.[7,8] However, many patients do not achieve desired LDL-C levels, and others experience side effects (eg, myalgia) that, despite being mild in the majority of the cases, may lead patients to abandon statin therapy or to take low and insufficient doses.[9,10] Medical misinformation with rapid spread over the Internet has also contributed to the abandonment of lifesaving therapies, such as statins.[11] Alternative therapies may be needed for those who cannot achieve the desired LDL-C levels or experience side effects leading to therapy low adherence and/or withdrawal.

The PCSK9 (proprotein convertase subtilisin/kexin type 9) is produced by the liver and secreted into the plasma, acting as a low-density lipoprotein receptor (LDLR) binder at the surface of the hepatocytes that prevents the recycling of the LDLR.[12] In consequence, the LDLR becomes more susceptible to degradation and less efficient in performing the clearance of the LDL-C, thereby increasing the circulating levels of LDL-C and the atherosclerosis risk.[13] Recent cardiovascular outcome trials have shown that PCSK9 inhibitors effectively reduce LDL-C, decrease the atheroma plaque burden, and reduce the rate of major cardiovascular events in high-risk patients with atherosclerotic cardiovascular disease and LDL-C levels of 70 mg/dL (1.8 mmol/L) or higher who were receiving statin therapy.[14,15] The development of PCSK9 inhibitor vaccines that are administered monthly or yearly have the potential to increase the treatment adherence and to substantially decrease the negative impact of atherosclerotic cardiovascular disease.[16] To date, no studies have accessed the potential role of PCSK9 inhibitors for primary cardiovascular prevention. *PCSK9* is a highly polymorphic gene, and some variants of the *PCSK9* gene are associated with variability in LDL-C.[17] Gain-of-function mutations interfere with the recycling of the LDLR, reducing the LDL-C uptake (and increasing LDL-C levels).[18] In particular, many studies have identified an association between the minor allele (A) of the rs562556 single-nucleotide polymorphism (SNP) (located on *PCSK9* gene, and responsible of missense mutation I474V) and LDL-C levels.[19]

The STANISLAS (Suivi Temporaire Annuel Non-Invasif de la Santé des Lorrains Assurés Sociaux) cohort is a single-center familial longitudinal cohort comprising 1006 families (4295 participants) from the Nancy region of France, who were recruited from 1993 to 1995 to visit 1. Participants were then followed each 5 to 10 years, and from the initial visit, 1705 participants returned for the fourth visit (visit 4), held from 2011 to 2016, which comprised a detailed cardiovascular assessment.[20] The circulating levels of cholesterol, PCSK9, and LDLR were assessed both at visit1 and visit 4; and the burden of atherosclerotic plaques was assessed at visit 4. A genome-wide association study was also performed on participants who participated to the visit 4. This design allows the determination on whether the PCSK9 and/or LDLR levels are associated with atherosclerotic plaques almost 20 years in advance, providing the background for a potential role of PCSK9 inhibitors in cardiovascular prevention. A potential causal link may be established if genetic variants associated with both PCSK9 and atherosclerotic plaques can be found.

The main aim of the present study in an initially healthy population is to study the association of PCSK9 and LDLR at visit 1 with carotid atherosclerotic plaques at visit 4, and the underlying genetic variants of *PCSK9* gene; therefore, hypothesizing a role for PCSK9 inhibitors in primary cardiovascular prevention.

Methods

Study Population

The data that support the findings of this study are available from the corresponding author upon reasonable request.

A detailed description of the STANISLAS cohort has been previously published.[20] The STANISLAS cohort was

established with the primary objective of investigating gene–gene and gene–environment interactions in the field of cardiovascular diseases. To assess the effect of genetics on the variability of intermediate phenotypes on the transition toward pathology, the families were deemed healthy and free of declared acute and/or chronic illness at visit 1. The implementation of the fourth visit enabled a follow-up of 18 to 23 years. The collected information was enriched with new biomarkers and detailed clinical phenotyping (including vascular echography). The STANISLAS visit 4 also allows the long-term evaluation of clinical, biological, and morphological data, such as the advent of atherosclerotic plaques in an initially healthy population.

Institutional Review Board approval was obtained, and all patients provided informed consent to participate in the study.

In the present analysis, we included the 997 adults who attended both visit 1 and visit 4, and had also performed vascular echography and biomarker, and 949 of them were successfully genotyped.

Study Design

All participants were observed at the Centre d'Investigation Clinique Plurithématique Pierre Drouin at Nancy Hospital Center (CIC-P de Nancy) in the morning after a 12- to 14-hour fast. Blood samples were taken. Medical history, medications, anthropometric parameters, blood pressure, carotid plaques, carotid-femoral pulse-wave velocity (PWV), and carotid intima-media thickness (cIMT) were recorded.

Arterial Plaques, Carotid Intima-Media Thickness, and PWV

High-resolution echotracking in STANISLAS visit 4 was performed to assess both carotid plaques, diameter, distention, and cIMT on the right common carotid artery.[21] Intima-media thickness was also measured in a subset of STANISLAS visit 1participants, but information regarding carotid plaques was not recorded.[22] The noninvasive investigations were performed in a controlled environment at 22±1°C after 10 minutes of rest in the supine position. Four measurements were obtained per each participant. Examinations were performed with the wall track system (ESOATE, Maastricht, The Netherlands) and the ART.LAB (ESAOTE, Maastricht, The Netherlands) in immediate succession. The reproducibility and agreement (intraoperator/interoperator/devices) of the measurements were excellent.

PWV was determined with the Complior (Complior SP, Alam Medical, France) and Sphygmocor CVMS (AtCor, Australia) devices. Peripheral blood pressure was measured after at least 10 minutes of rest in the supine position, in a quiet room. Carotid-to-femoral PWV was assessed with Complior using the recommendations of the European Network for Noninvasive Investigation of Large Arteries.[23]

Biomarkers and Gene-Candidate Analysis

All samples were collected at the Centre d'Investigation Clinique Plurithématique Pierre Drouin at Nancy Hospital Center with minimally traumatic venipuncture. Standardized sample-handling procedures enabled the collection of serum and plasma (EDTA, heparin) as well as buffy coat fraction. Blood DNA of all the participants to the STANISLAS visit 4 was extracted using Gentra Puregene Blood Kit (Qiagen, Hilden, Germany) and stored at −20°C. Genotyping was conducted at the Centre National de Recherche en Génomique Humaine (Evry, France) using 2 chips: the Illumina Global Screening Array, which is composed of 687 572 intronic and exonic markers and the Illumina Exome Array, which is composed of 244 330 SNPs, mostly exonic. All blood-derived biosamples are stored in a central biobank facility with temperatures between −80°C and −196°C (as required).

Baseline plasma samples were analyzed for protein biomarkers by the TATAA-biocenter using the Olink Proseek Multiplex cardiovascular II panel that includes both PCSK9 and LDLR, using a proximity extension assay technology,[24] where 92 oligonucleotide-labeled antibody probe pairs per panel are allowed to bind to their respective targets in the sample in 96-well plate format. When binding to their correct targets, they give rise to new DNA amplicons, with each ID-barcoding their respective antigens. The amplicons are subsequently quantified using a BioMark HD real-time polymerase chain reaction platform (Fluidigm, San Francisco, CA). The platform provides $\log_2$-normalized protein expression data.

For the genetic analyses of the present study, we focused on the *PCSK9* gene, which encodes the PCSK9 protein. First, we defined an interval that encompassed the *PCSK9* gene boundaries (±20 kb) on chromosome 1 based on the reference genome built 37 from the Ensembl database (Chr1; position 55485221-55550525; http://grch37.ensembl.org). Then, we selected all the SNPs comprised between these boundaries in the 2 chips. Twenty-four SNPs were selected from the Global Screening Array chip and 12 from the Exome chip. However, 3 markers were duplicated between the 2 chips, and 1 of each was excluded. Hence, 33 SNPs were selected. After the quality control steps, 1 SNP was excluded for monomorphism (ie, minor allele frequency=0); no SNP had >1% of missing data (ie, all SNPs had a call rate >0.99), and no SNP deviated from the Hardy–Weinberg equilibrium at a threshold of $P<1.10^{-8}$. We also excluded 7 SNPs that are rare (minor allele frequency <0.01). Tests for linkage disequilibrium in the subset of the 26 SNPs were conducted, and 4 SNPs were highly linked with $r^2>0.90$.

Statistical Analysis

For the baseline clinical characteristics, continuous variables are expressed as means and respective SD. Categorical variables are presented as frequencies and percentages. Participant baseline characteristics were compared between those without plaques versus those with plaques at visit 4 using chi-squared tests for categorical variables and t tests for continuous variables.

The main aim of this study was to test the association of PCSK9 and LDLR with incident carotid plaques. Logistic regression models were performed. First, a stepwise backward model including all the clinical variables with a $P<0.1$ from Table 1 was performed, to select the clinical features with stronger association with carotid plaques. Second, the potential association of PCSK9 and LDLR with carotid plaques was tested on top of the clinical model built in the previous step. We also tested the association of the genetic alleles on top of the clinical model plus PCSK9 and LDLR. The stability of this model was also confirmed using "partialing-out cross-fit" estimators controlling for all the variables with a $P<0.01$ from Table 1 with overlapping results (data not shown).

Since PCSK9 and LDLR proteins were measured using normalized protein expression) values on a $\log_2$ scale, the odds ratio (OR) for each protein estimates the increase in the odds of carotid plaques associated with a doubling in the protein concentration. A $P<0.05$ was considered statistically significant. The analyses were performed using STATA version 15 software (Stata Statistical Software, Release 15, StataCorp LP, College Station, TX).

The genetic analyses were performed using R (version 3.4.1). Association tests were performed using the R package "gaston."[25] The association of the genetic variants with the study outcomes was tested using a linear model, with age and sex used as covariates. The statistical significance level was fixed at 0.05, after applying a Benjamini–Hochberg correction for multiple testing.

Results

Characteristics of the Population

In the present analyses 997 adult participants were included. The mean age of the adult population at visit 1 was 42±5 years. Of these, 203 (20.4%) had carotid plaque(s) at visit 4. Participants who developed arterial plaques (from visit 1 to visit 4) were older than the other participants (42.7±5.4 versus 41.7±4.7 years), more often male (60% versus 49%), smokers (29% versus 18%), with diabetes mellitus (6% versus 3%), and with higher cholesterol levels (LDL-C 1.6±0.4 versus 1.5±0.3 g/L) ($P<0.05$ for all) at visit 1 (Table 1). Participants who developed arterial plaques also had higher circulating PCSK9 and LDLR levels (Table 1).

Table 1. Baseline (Visit 1) Characteristics of the Study Population by the Presence of Carotid Plaques at Visit 4

Patients' Characteristics at Visit 1	No Plaque	Plaque at Visit 4	*P* Value
N. total=997	794	203	
Age, y	41.7±4.7	42.7±5.4	0.009
Male sex	387 (48.7%)	122 (60.1%)	0.004
BMI, kg/m^2	24.3±3.7	24.7±3.8	0.22
Waist circumference, cm	80.6±11.1	83.6±11.7	<0.001
Smoking	140 (18.4%)	58 (28.6%)	0.002
SBP, mm Hg	122.1±11.8	124.4±13.6	0.015
DBP, mm Hg	74.4±9.8	75.6±10.3	0.11
Heart rate, bpm	66.3±10.3)	64.7±10.1	0.056
Diabetes mellitus	21 (2.8%)	12 (5.9%)	0.028
Glucose, g/L	0.9±0.1	0.9±0.1	0.13
Hypertension history	69 (9.1%)	25 (12.4%)	0.16
Total cholesterol, g/L	2.2±0.4	2.3±0.4	0.010
HDL cholesterol, g/L	0.6±0.2	0.5±0.2	0.045
LDL cholesterol, g/L	1.5±0.3	1.6±0.4	0.001
Triglycerides, g/L	0.9±0.6	1.1±1.5	0.011
Lipid-lowering therapy	13 (5.3%)	3 (4.5%)	0.79
eGFR, mL/min per 1.73 m^2	90.2±12.3	90.4±14.1	0.84
PCSK9 (NPX)	2.7±0.4	2.8±0.4	<0.001
LDL receptor (NPX)	5.2±0.6	5.3±0.6	0.012

Median time between visit 1 and visit 4 , 18.5 years. BMI indicates body mass index; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate calculated by the Chronic Kidney Disease Epidemiology Collaboration formula; LDL, low-density lipoprotein; NPX, Olink $\log_2$ normalized protein expression; PCSK9, proprotein convertase subtilisin/kexin type 9; SBP, systolic blood pressure.

The median (pct_{25-75}) follow-up time from visit 1 to visit 4 was 18.5 (17.7–19.7) years. The mean age at visit 4 was 59.2±5.7 years, and the characteristics of the population with versus without plaques at visit 4 is described in the Table S1. No patient had carotid artery stenosis >50% of the lumen diameter.

Factors Associated With Carotid Plaques

The independent clinical factors at visit 1 associated with the advent (~20 years after) of arterial plaques at visit 4, were age (OR] per 5-year increase, 1.26; 95% CI, 1.06–1.49; $P=0.007$), smoking (OR, 1.79; [95% CI, 1.23–2.60; $P=0.002$), and LDL-C (OR per 10 mg/dL increase, 1.72; 95% CI, 1.10–2.69; $P=0.018$) (Table 2). PCSK9 at visit 1 was associated with

visit 4 arterial plaques on top of the clinical model (OR, 2.14, 95% CI, 1.28–3.58; *P*=0.004) and LDLR was not (OR, 0.91; 95% CI, 0.66–1.25; *P*=0.57). The PCSK9/LDLR ratio was also associated with arterial plaques driven by the PCSK9 levels (OR, 1.32; 95% CI, 1.04–1.67; *P*=0.023) (Table 2 and Figure. PCSK9 levels at visit 4 was also "transversally" associated with carotid plaques at visit 4 (OR, 1.66; 95% CI, 1.01–2.72; *P*=0.045) (Table S2).

Sensitivity Analysis in Children at Baseline

The proportion of children at visit 1 (n=647; mean age, 15±4 years) who developed plaques at visit 4 (mean age, 33±5 years) was 0.9% (n=6) (Table S3 and Figure S1). Despite an almost 10-year difference, the age of these participants at visit 4 was closer to their parents at visit 1, possibly indicating that our population might have low prevalence of carotid plaques at visit 1.

Association of PCSK9 and LDLR With cIMT and PWV

At visit 1 LDLR was independently associated with visit 4 PWV as continuous variable (β-coefficient, 0.4; 95% CI, 0.1–0.6; *P*=0.006). At visit 4, LDLR was independently associated with visit 4 cIMT as a continuous and categorical variable (β-coefficient, 20.9; 95% CI, 4.5–37.2; *P*=0.012; and OR, 1.32; 95% CI, 1.00–1.73; *P*=0.042 for cIMT above the 90th percentile, respectively) (Table S4).

Table 2. Predictors (Visit 1) of Carotid Artery Plaque (at Visit 4)

Variable	OR (95% CI)	*P* Value
"Best" clinical model		
Age (per 5 y)	1.26 (1.06–1.49)	0.007
Smoking (active)	1.79 (1.23–2.60)	0.002
LDL-C, g/L	1.72 (1.10–2.69)	0.018
PCSK9 plus LDLR on top of the "best" clinical model		
PCSK9 (NPX)	2.14 (1.28–3.58)	0.004
LDLR (NPX)	0.91 (0.66–1.25)	0.57
rs562556 polymorphism on top of the "best" clinical model plus PCSK9 and LDLR proteins		
rs562556 (risk per A allele)	1.60 (1.10–2.32)	0.014
PCSK9/LDLR ratio on top of the "best" clinical model		
PCSK9/LDLR (ratio)	1.32 (1.04–1.67)	0.023

N=997; N. Plaque=203. Median time between visit 1 and visit 4 , 18.5 years. Our models presented good fit: Hosmer –Lemeshow goodness-of-fit test *P*>0.5 for all models (ie, clinical alone and clinical plus biomarkers). LDL-C indicates low-density lipoprotein cholesterol; LDLR, low-density lipoprotein receptor; NPX, Olink $\log_2$ normalized protein expression; OR, odds ratio; PCSK9, proprotein convertase subtilisin/kexin type 9.

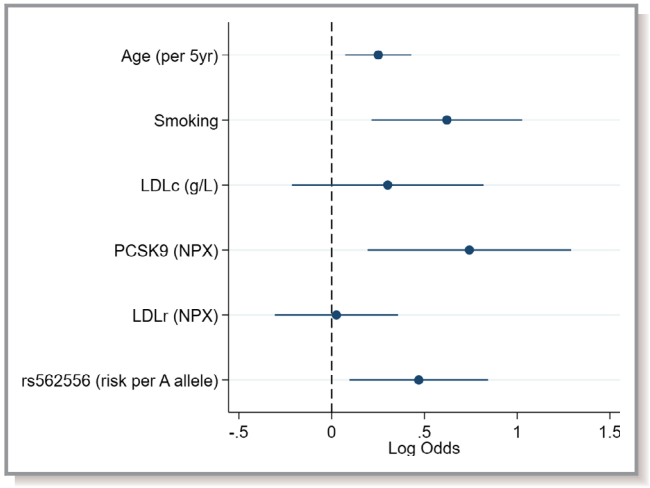

Figure. Multivariable predictors (visit 1) of carotid artery plaque (at visit 4). N =997; N. Plaque =203. Median time between visit 1 and visit 4, 18.5 years. LDL-C indicates low-density lipoprotein cholesterol; LDLR, LDL receptor; NPX, Olink $\log_2$ normalized protein expression; PCSK9, proprotein convertase subtilisin/kexin type 9.

Genetic Considerations

We tested the association between polymorphisms of *PCSK9* gene at visit 1 and presence of atherosclerotic plaques at visit 4 (Figure S2). We found 3 SNPs (rs540796, rs562556, and rs631220) associated with the presence of plaques (corrected *P*=0.02; Table S5). Minor alleles of these 3 SNPs are also associated with LDL-C and PCSK9 levels both at visit 1 and visit 4. These 3 SNPs are highly correlated among them (r^2>0.9) and 1 of them (rs562556) carried a missense gain-of-function mutation (I474V). Subjects who carried the minor allele (genotypes AA or GA) had higher cholesterol level and more often carotid plaques (Tables S6, S7, S8, S9, and S10). However, no significant association was found between *PCSK9* polymorphisms and PWV or cIMT.

The partial correlation analyses suggest that rs562556 contributes with 16% to 17% of the variance of PCSK9 levels both at visit 1 and visit 4 (*P*<0.001 for both; (Table S11).

Discussion

As the main finding, the present study shows that increased circulating levels of PCSK9 in middle-aged healthy people were independently associated with the presence of carotid plaques 20 years later, adjusting for important contributors of carotid plaque formation (eg, age, smoking, LDL-C). Moreover, PCSK9 levels were similarly associated with carotid plaques "transversally" at visit 4. The identification of the SNP rs562556 (responsible for the missense mutation I474V) provides a potential causal link between the PCSK9 levels and the atherosclerotic plaques.

Higher LDL-C concentrations have causal association with increased cardiovascular risk. Evidence derived from multiple randomized controlled trials and meta-analyses shows a consistent and graded reduction in cardiovascular risk in response to reductions in the LDL-C levels (grade of evidence IA).[8,26–29] There is no apparent threshold at which LDL-C lowering is not associated with reduced cardiovascular risk.[8] Moreover, the higher the initial LDL-C level, the greater the absolute reduction in risk, while the relative risk reduction remains constant at any given baseline LDL-C level.[30,31] Statins effectively lower LDL-C and are generally well tolerated. The only (rare) adverse events that have been reliably shown to be caused by statins are myalgias.[32] However, many patients are reluctant in taking statins, many because of medical misinformation, while many others cannot achieve the desired levels of LDL-C despite moderate- to high-intensity statin therapy.[11]

The *PCSK9, LDLR,* and apolipoprotein B genes have been associated with autosomal dominant forms of familial hypercholesterolemia, caused by a "gain-of-function" mutation in the *PCSK9* gene, resulting in excessive LDL-C and, consequently, atherosclerotic plaques and cardiovascular events.[33] Mutations in the *PCSK9* gene are reported to be responsible for 10% to 25% of the autosomal dominant form of familial hypercholesterolemia cases without mutations in LDLR or apolipoprotein B.[34] On the other hand, individuals who have a "loss-of-function" mutation in the *PCSK9* gene express lower levels of LDL-C and have low cardiovascular risk.[35] The physiological explanation is that the circulating PCSK9 binds to the LDLR, increasing its degradation in the lysosomal compartments. Inhibiting the PCSK9 activity (eg, with a monoclonal antibody) results in lower concentrations of free PCSK9, and in consequence, fewer LDLRs are degraded, thus being available for the uptake of LDL-C, decreasing its blood concentrations.[36] The advent of PCSK9 inhibitors has enabled their testing in phase 3 trials. Overall, PCSK9 inhibitors significantly reduced LDL-C regardless of patient population and/or background statin treatment.[37,38]

Previous to the present study, evidence regarding an independent association of PCSK9 in atherosclerosis progression was scarce. Prior cross-sectional studies reported conflicting results regarding the association between PCSK9 levels and cIMT.[39,40] In concordance with our results, in a Chinese cohort of 643 participants free of cardiovascular disease at baseline, plasma PCSK9 levels were associated with 10-year progression of atherosclerosis (measured by the total plaque area), independently from LDL.[41] However, in this study, the association of polymorphisms of the *PCSK9* gene and the progression of atherosclerotic plaques were not evaluated.

In our study, we found that the rs562556 missense mutation was independently associated with incident carotid atherosclerotic plaques, LDL-C, and PCSK9 levels. The SNP rs562556 has already been found to be associated with LDL-C and total cholesterol, whereby patients with the mutation have higher levels of cholesterol.[19] Another recent study showed that genetically low LDL-C attributable to PCSK9 variation (including the rs562556 SNP) was causally associated with low risk of cardiovascular mortality but not with low all-cause mortality in a general population.[42] These findings are in agreement with our results.

Our models retained age, smoking, and LDL-C as the factors with stronger association with the occurrence of plaques, in concordance with previous studies.[43] Hence, the independent association of PCSK9 on top of these "classic" risk markers reinforces our hypothesis of targeting PCSK9 for preventing the occurrence of atherosclerotic disease. As described in the introductory section, the presence of atherosclerotic plaques has been associated with the occurrence of major cardiovascular events beyond cIMT or PWV.[2,4,44]

Limitations

Several limitations should be acknowledged in the present study. First, this is an observational study; therefore, no causality can be established. Second, the presence of plaques at visit 1 was not recorded; therefore, we cannot exclude that some of these patients already had carotid plaques in their 40s; however, the near absence of plaques at visit 4 in those who started the study visit 1 as teenagers provides a robust internal control. Third, PCSK9 and LDLR were measured with the Olink technology standardized $\log_2$ normalized protein expression values, and no direct conversion to the standard "mass" values is possible.

Conclusions

PCSK9 was associated with arterial plaques almost 20 years in advance in a healthy middle-aged population. The association of plaque and the SNP rs562556, responsible for I474V mutation, may reinforce the potential causality of our findings. Whether PCSK9 inhibitors could be useful for primary cardiovascular prevention could be worth investigating.

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Disclosures

The authors declare not having conflicts of interest with regards to the content of this manuscript.

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Supplemental Material

Table S1. Characteristics of the study population (at visit 4) by the presence of carotid plaques at visit 4.

Patients` characteristics at V4	No plaque	Plaque at V4	p-value
N. total =997	794	203	
Age (yr)	58.7 ± 5.8	61.4 ± 5.2	<0.001
Male sex	387 (48.7%)	122 (60.1%)	0.004
BMI (Kg/m2)	26.9 ± 4.8	27.4 ± 4.8	0.16
Waist circumference (cm)	92.6 ± 13.3	95.7 ± 13.6	0.003
Smoking	99 (12.5%)	29 (14.4%)	0.46
SBP (mmHg)	128.2 ± 15.5	134.4 ± 16.9	<0.001
DBP (mmHg)	74.3 ± 8.8	74.4 ± 9.0	0.87
Heart rate (bpm)	63.5 ± 8.9	62.7 ± 10.5	0.28
Diabetes	57 (7.3%)	21 (10.7%)	0.27
Glucose (g/L)	0.9 ± 0.2	1.0 ± 0.2	<0.001
Hypertension history	225 (28.4%)	76 (37.8%)	0.010
Total cholesterol (g/L)	2.2 ± 0.4	2.2 ± 0.4	0.46
HDL cholesterol (g/L)	0.6 ± 0.1	0.6 ± 0.1	0.59
LDL cholesterol (g/L)	1.4 ± 0.3	1.4 ± 0.4	0.11
Triglycerides (g/L)	1.1 ± 0.8	1.2 ± 0.6	0.033
Lipid lowering therapy	181 (22.8%)	71 (35.0%)	<0.001
eGFR (ml/min)	88.3 ± 12.0	89.1 ± 12.9	0.41
Carotid stenosis 20-50%	0 (0.0%)	15 (100.0%)	<0.001
cIMT (µm)	691 ± 138	722 ± 132	0.005
PWV (m/s)	9.0 ± 1.7	9.8 ± 2.1	<0.001
PCSK9 (NPX)	2.9 ± 0.4	3.0 ± 0.4	<0.001
LDL receptor (NPX)	5.5 ± 0.6	5.6 ± 0.5	0.016

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate calculated by the CKD-EPI formula; LDL, low-density lipoprotein; cIMT, carotid intima-media thickness; PWV, pulse-wave velocity; PCSK9, proprotein convertase subtilisin/kexin type 9; NPX, Olink® log2 standardized units.

Table S2. Factors associated with carotid artery plaque at visit 4.

Variable	OR (95%CI)	p-value
"Best" clinical model		
Age (per 5 yr)	1.48 (1.26-1.73)	<0.001
SBP (per 10 mmHg)	1.18 (1.07-1.30)	0.001
Lipid-lowering therapy	1.39 (0.98-1.97)	0.062
PCSK9 plus LDLr on top of the "best" clinical model		
PCSK9 (NPX)	1.66 (1.01-2.72)	0.045
LDLr (NPX)	1.12 (0.82-1.53)	0.48
PCSK9/LDLr ratio on top of the "best" clinical model		
PCSK9/LDLr (ratio)	1.13 (0.89-1.43)	0.33

N =997; N. Plaque =203.
LDLc, low-density lipoprotein cholesterol; PCSK9, proprotein convertase subtilisin/kexin type 9; LDLr, LDL receptor; NPX, Olink® log2 standardized units.

Table S3. Plaque at visit 4 in the "children".

Children at V1	No plaque	Plaque at V4
N. total =647	641 (99.1%)	6 (0.9%)

Table S4. Association of PCSK9 and LDLr (visit 1 and visit 4) with IMT and PWV (at visit 4).

Visit 1 (PCSK9 and LDLr)		
IMT above the 90th percentile	**OR (95%CI)**	**p-value**
PCSK9 (NPX)	1.03 (0.67-1.61)	0.87
LDLr (NPX)	1.11 (0.84-1.47)	0.45
IMT (mm) continuous	**Coef. (95%CI)**	**p-value**
PCSK9 (NPX)	16.1 (-10.7 to +42.8)	0.24
LDLr (NPX)	11.8 (-5.2 to +28.7)	0.18
PWV above the 90th percentile	**OR (95%CI)**	**p-value**
PCSK9 (NPX)	1.01 (0.46-2.20)	0.98
LDLr (NPX)	1.23 (0.75-2.01)	0.41
PWV (m/s) continuous	**Coef. (95%CI)**	**p-value**
PCSK9 (NPX)	-0.1 (-0.4 to +0.3)	0.60
LDLr (NPX)	0.4 (+0.1 to +0.6)	0.002
Visit 4 (PCSK9 and LDLr)		
IMT above the 90th percentile	**OR (95%CI)**	**p-value**
PCSK9 (NPX)	0.87 (0.58-1.30)	0.51
LDLr (NPX)	1.32 (1.00-1.73)	0.042
IMT (mm) continuous	**Coef. (95%CI)**	**p-value**
PCSK9 (NPX)	-2.9 (-27.4 to +21.5)	0.81
LDLr (NPX)	20.9 (+4.5 to +37.2)	0.012
PWV above the 90th percentile	**OR (95%CI)**	**p-value**
PCSK9 (NPX)	1.09 (0.55-2.18)	0.79
LDLr (NPX)	1.17 (0.74-1.84)	0.51
PWV (m/s) continuous	**Coef. (95%CI)**	**p-value**
PCSK9 (NPX)	0.1 (-0.3 to +0.3)	0.92
LDLr (NPX)	0.1 (-0.1 to +0.3)	0.27

Model adjusted on age, sex, smoking and LDLc.

Table S5. Association between presence of plaques and PCSK9 polymorphisms (n=949).

id	Allele 1	Allele 2	freqA2	beta	sd	p	BH
rs2479394	G	A	0.702	0.124	0.132	0.348	0.566
rs11588151	G	A	0.778	0.151	0.151	0.318	0.566
rs11206510	C	T	0.795	0.256	0.157	0.103	0.269
rs17192725	A	G	0.900	0.013	0.200	0.950	0.950
rs2182833	G	A	0.800	-0.141	0.150	0.348	0.566
rs2479409	G	A	0.655	-0.281	0.125	0.025	0.130
rs11591147	T	G	0.985	0.120	0.513	0.814	0.847
rs11583680	T	C	0.856	0.409	0.190	0.032	0.137
rs499718	T	C	0.815	0.101	0.159	0.524	0.632
rs28385708	T	C	0.932	0.163	0.245	0.507	0.632
rs41294821	T	C	0.990	-0.559	0.545	0.305	0.566
rs7552841	T	C	0.600	0.037	0.124	0.768	0.832
rs58255540	G	A	0.948	-0.209	0.255	0.412	0.630
rs634272	T	C	0.741	0.412	0.147	0.005	0.032
rs11808052	T	C	0.948	-0.134	0.259	0.604	0.683
rs540796	A	G	0.838	0.578	0.188	0.002	0.024
rs562556	G	A	0.838	0.584	0.188	0.002	0.024
rs114162366	A	G	0.981	-0.301	0.403	0.455	0.632
rs631220	A	G	0.838	0.562	0.187	0.003	0.024
rs505151	G	A	0.968	0.248	0.377	0.510	0.632
rs41294827	T	G	0.973	-0.653	0.333	0.050	0.143
rs77011887	T	C	0.979	-0.443	0.386	0.251	0.544
rs77868073	C	T	0.974	-0.673	0.334	0.044	0.142
rs487230	A	G	0.784	0.327	0.159	0.039	0.142
rs114527615	A	G	0.980	0.289	0.466	0.535	0.632
rs17111584	C	T	0.968	-0.487	0.309	0.114	0.271

Table S6. Crude odd ratios for association between plaques at V4 and genotype at rs562556.

	Plaques (n=184)	No plaques (n=718)	OR (95%CI) (risk per A allele)	P trend
Rs562556				
GG	0	24		
GA	41	201	1.67 (1.17-2.38)	P = 0.004
AA	143	493		

Table S7. Association between *PCSK9* polymorphisms and circulating LDL at visit 4 (n=895).

id	Allele 1	Allele 2	freqA2	beta	sd	p	BH
rs2479394	G	A	0.703	-0.019	0.017	0.273	0.671
rs11588151	G	A	0.777	-0.010	0.019	0.617	0.895
rs11206510	C	T	0.794	-0.017	0.020	0.382	0.671
rs17192725	A	G	0.899	-0.013	0.026	0.619	0.895
rs2182833	G	A	0.800	-0.016	0.020	0.416	0.675
rs2479409	G	A	0.654	-0.006	0.017	0.703	0.918
rs11591147	T	G	0.985	0.080	0.065	0.218	0.671
rs11583680	T	C	0.856	-0.025	0.023	0.269	0.671
rs499718	T	C	0.815	-0.007	0.020	0.747	0.925
rs28385708	T	C	0.931	-0.027	0.031	0.387	0.671
rs41294821	T	C	0.990	-0.004	0.079	0.964	0.964
rs7552841	T	C	0.601	-0.018	0.016	0.269	0.671
rs58255540	G	A	0.948	0.032	0.035	0.358	0.671
rs634272	T	C	0.741	-0.007	0.018	0.706	0.918
rs11808052	T	C	0.948	0.033	0.035	0.343	0.671
rs540796	A	G	0.837	0.053	0.021	0.012	0.088
rs562556	G	A	0.837	0.052	0.021	0.014	0.088
rs114162366	A	G	0.980	-0.005	0.057	0.929	0.964
rs631220	A	G	0.838	0.053	0.021	0.011	0.088
rs505151	G	A	0.968	-0.006	0.045	0.888	0.964
rs41294827	T	G	0.973	0.069	0.049	0.161	0.671
rs77011887	T	C	0.979	-0.005	0.055	0.930	0.964
rs77868073	C	T	0.974	0.063	0.049	0.202	0.671
rs487230	A	G	0.783	0.048	0.019	0.013	0.088
rs114527615	A	G	0.980	-0.013	0.056	0.815	0.963
rs17111584	C	T	0.968	0.042	0.044	0.340	0.671

Table S8. Association between *PCSK9* polymorphisms and circulating LDL at visit 1 (n=861).

id	Allele 1	Allele 2	freqA2	beta	sd	p	BH
rs2479394	G	A	0.699	-0.011	0.018	0.534	0.816
rs11588151	G	A	0.776	0.050	0.020	0.015	0.077
rs11206510	C	T	0.793	0.033	0.021	0.113	0.327
rs17192725	A	G	0.899	-0.056	0.028	0.042	0.156
rs2182833	G	A	0.797	-0.025	0.021	0.248	0.538
rs2479409	G	A	0.653	-0.034	0.018	0.054	0.176
rs11591147	T	G	0.985	0.190	0.068	0.005	0.077
rs11583680	T	C	0.856	0.012	0.024	0.605	0.828
rs499718	T	C	0.814	-0.006	0.021	0.778	0.963
rs28385708	T	C	0.932	0.007	0.033	0.824	0.974
rs41294821	T	C	0.990	0.025	0.084	0.765	0.963
rs7552841	T	C	0.598	-0.024	0.017	0.155	0.393
rs58255540	G	A	0.948	0.029	0.037	0.439	0.761
rs634272	T	C	0.744	0.021	0.019	0.274	0.549
rs11808052	T	C	0.948	0.030	0.037	0.427	0.761
rs540796	A	G	0.838	0.057	0.022	0.011	0.077
rs562556	G	A	0.837	0.056	0.022	0.012	0.077
rs114162366	A	G	0.983	0.000	0.064	0.996	0.996
rs631220	A	G	0.839	0.059	0.022	0.009	0.077
rs505151	G	A	0.969	-0.112	0.048	0.021	0.090
rs41294827	T	G	0.974	0.007	0.053	0.897	0.996
rs77011887	T	C	0.979	-0.032	0.059	0.589	0.828
rs77868073	C	T	0.975	-0.001	0.054	0.984	0.996
rs487230	A	G	0.787	0.028	0.020	0.166	0.393
rs114527615	A	G	0.980	0.039	0.059	0.511	0.816
rs17111584	C	T	0.971	-0.004	0.050	0.935	0.996

Table S9. Association between *PCSK9* polymorphisms and circulating PCSK9 at visit 1 (n=894).

id	Allele 1	Allele 2	freqA2	beta	sd	p	BH
rs2479394	G	A	0.702	-0.026	0.019	1.7E-01	2.9E-01
rs11588151	G	A	0.777	0.061	0.021	3.8E-03	9.5E-03
rs11206510	C	T	0.794	0.061	0.021	4.4E-03	9.5E-03
rs17192725	A	G	0.899	-0.038	0.028	1.8E-01	2.9E-01
rs2182833	G	A	0.800	-0.077	0.022	4.6E-04	1.3E-03
rs2479409	G	A	0.655	-0.048	0.018	7.7E-03	1.5E-02
rs11591147	T	G	0.985	0.326	0.070	3.4E-06	1.7E-05
rs11583680	T	C	0.855	0.120	0.024	8.8E-07	1.5E-05
rs499718	T	C	0.814	0.087	0.022	7.7E-05	2.5E-04
rs28385708	T	C	0.931	-0.023	0.034	5.1E-01	6.0E-01
rs41294821	T	C	0.990	0.053	0.086	5.4E-01	6.0E-01
rs7552841	T	C	0.601	-0.050	0.017	4.2E-03	9.5E-03
rs58255540	G	A	0.949	0.027	0.038	4.9E-01	6.0E-01
rs634272	T	C	0.740	0.089	0.019	4.0E-06	1.7E-05
rs11808052	T	C	0.949	0.031	0.038	4.2E-01	5.8E-01
rs540796	A	G	0.837	0.110	0.023	1.8E-06	1.5E-05
rs562556	G	A	0.837	0.110	0.023	1.7E-06	1.5E-05
rs114162366	A	G	0.980	-0.053	0.062	4.0E-01	5.7E-01
rs631220	A	G	0.837	0.108	0.023	2.5E-06	1.6E-05
rs505151	G	A	0.968	-0.063	0.049	2.1E-01	3.1E-01
rs41294827	T	G	0.973	0.023	0.054	6.7E-01	7.2E-01
rs77011887	T	C	0.979	-0.042	0.060	4.8E-01	6.0E-01
rs77868073	C	T	0.974	0.021	0.054	7.0E-01	7.3E-01
rs487230	A	G	0.783	0.083	0.021	7.3E-05	2.5E-04
rs114527615	A	G	0.980	0.120	0.061	5.1E-02	9.5E-02
rs17111584	C	T	0.968	0.002	0.049	9.7E-01	9.7E-01

Table S10. Association between *PCSK9* polymorphisms and circulating PCSK9 at visit 4 (n=897).

id	A1	A2	freqA2	beta	sd	p	BH
rs2479394	G	A	0.702	-0.016	0.018	3.94E-01	5.12E-01
rs11588151	G	A	0.778	0.079	0.021	1.28E-04	3.70E-04
rs11206510	C	T	0.795	0.101	0.021	1.62E-06	1.05E-05
rs17192725	A	G	0.900	-0.031	0.028	2.68E-01	3.69E-01
rs2182833	G	A	0.800	-0.052	0.022	1.59E-02	2.96E-02
rs2479409	G	A	0.655	-0.065	0.018	2.63E-04	6.84E-04
rs11591147	T	G	0.985	0.429	0.068	5.46E-10	1.42E-08
rs11583680	T	C	0.856	0.143	0.024	3.75E-09	4.88E-08
rs499718	T	C	0.815	0.084	0.022	1.13E-04	3.67E-04
rs28385708	T	C	0.931	0.024	0.033	4.67E-01	5.78E-01
rs41294821	T	C	0.990	0.094	0.085	2.70E-01	3.69E-01
rs7552841	T	C	0.599	-0.053	0.017	2.23E-03	5.26E-03
rs58255540	G	A	0.949	0.091	0.038	1.78E-02	3.08E-02
rs634272	T	C	0.741	0.096	0.019	5.09E-07	4.41E-06
rs11808052	T	C	0.949	0.090	0.038	2.00E-02	3.25E-02
rs540796	A	G	0.838	0.103	0.023	6.34E-06	2.75E-05
rs562556	G	A	0.838	0.105	0.023	3.93E-06	2.04E-05
rs114162366	A	G	0.980	0.022	0.062	7.26E-01	7.55E-01
rs631220	A	G	0.839	0.102	0.023	7.68E-06	2.85E-05
rs505151	G	A	0.968	-0.139	0.049	4.51E-03	9.76E-03
rs41294827	T	G	0.973	0.031	0.053	5.52E-01	6.24E-01
rs77011887	T	C	0.979	0.015	0.059	7.98E-01	7.98E-01
rs77868073	C	T	0.974	0.035	0.053	5.16E-01	6.09E-01
rs487230	A	G	0.784	0.058	0.021	5.23E-03	1.05E-02
rs114527615	A	G	0.980	0.068	0.061	2.63E-01	3.69E-01
rs17111584	C	T	0.968	0.023	0.048	6.35E-01	6.88E-01

Table S11. Partial correlation coefficients.

Variable	Partial correlation	P-value
PCSK9 levels at V1		
Age at V1	-5.2%	0.12
Smoking at V1	-0.1%	0.77
LDLc at V1	15.0%	<0.001
LDLr at V1	37.6%	<0.001
rs562556	16.0%	<0.001
PCSK9 levels at V4		
Age at V4	-2.3%	0.49
SBP at V4	-1.5%	0.65
Statin at V4	30.0%	<0.001
LDLr at V4	36.5%	<0.001
rs562556	16.5%	<0.001

Figure S1. Distribution of the Stanislas cohort population according to age.

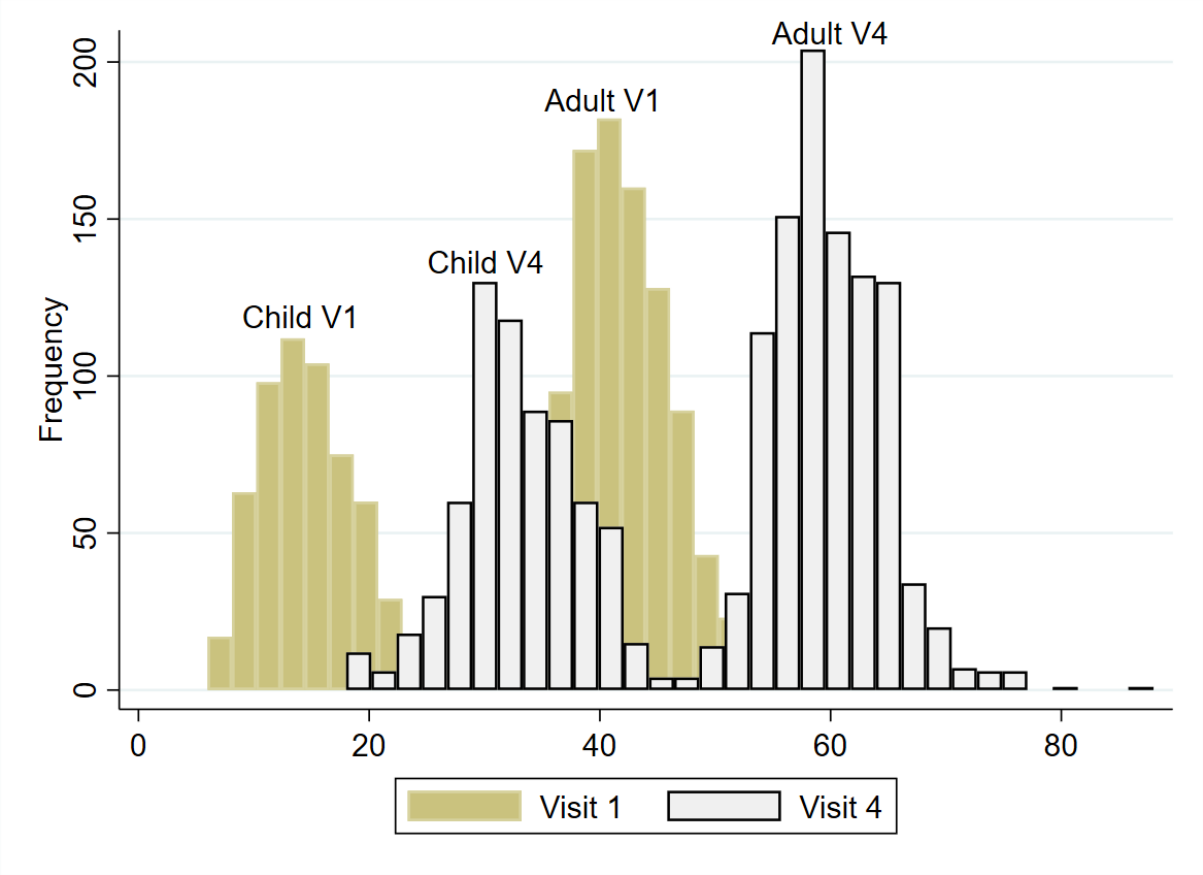

Figure S2. Matrix of linkage disequilibrium of the 26 SNPs for the gene *PCSK9*.