Plasma Proprotein Convertase Subtilisin/Kexin Type 9 as a Predictor of Carotid Atherosclerosis in Asymptomatic Adults

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Abstract

Background: Atherosclerosis is a lipid-driven inflammatory disease of the arterial wall involving complex and multifactorial processes. Proprotein convertase subtilisin kexin type 9 (PCSK9) may play a role in the development of atherosclerosis.

Methods: We investigated the associations between serum PCSK9 and carotid intima-medial wall thickness (IMT), a measure of subclinical atherosclerosis that predicts cardiovascular events, in 295 asymptomatic subjects from community. Carotid IMT was determined by high-resolution B-mode carotid ultrasonography and serum PCSK9 was measured by immunoassay.

Results: In univariate analysis, serum PCSK9 concentration was positively ($P<0.05$ in all) associated with age ($r=0.204$), BMI ($r=0.149$), waist circumference ($r=0.139$), systolic blood pressures ($r=0.116$), glucose ($r=0.211$), insulin ($r=0.178$), HOMA score ($r=0.195$), plasma triglyceride ($r=0.285$), total cholesterol ($r=0.241$) and LDL-cholesterol concentrations ($r=0.172$). In multivariate regression including male gender, hypertension, smoking status, HOMA score, obesity, LDL-cholesterol, lipoprotein (a) or markers of inflammation, serum PCSK9 remained an independent predictor of mean carotid IMT ($P<0.001$).

Conclusions: These data suggest that serum levels of PCSK9 may contribute to increased risk of subclinical carotid atherosclerosis independent of conventional risk factors. Whether PCSK9 inhibition improves cardiovascular outcomes remains to be demonstrated in large, ongoing clinical trials.

Keywords: PCSK9, Cartoid IMT and Cardiovascular Disease

INTRODUCTION

Atherosclerosis is a chronic, lipid-driven inflammatory disease of the arterial wall involving complex and multifactorial processes, such as endothelial dysfunction, influx and modification of low-density lipoprotein (LDL), leukocyte recruitment, foam cell formation and plaque development [1]. Individuals with subclinical atherosclerosis are at increased risk for future cardiovascular disease (CVD) [2, 3]. Although traditional plasma lipid and inflammatory factors are important for the development of atherosclerosis, they do not fully account for the variation in risk of CVD. Hence, identifying new plasma markers associated with subclinical atherosclerosis can have important application for clinical practice.

Proprotein convertase subtilisin kexin type 9 (PCSK9), a secretory protease produced by the liver and detectable in human plasma, has recently been suggested to play a role in the development of atherosclerosis [4]. PCSK9 is a key regulator of the LDL receptor and hence the metabolism of LDL [5]. In vitro and animal studies demonstrate that secreted PCSK9 binds to and redirects the LDL receptor to lysosomes for degradation; this inhibits the intracellular recycling of LDL receptors and the subsequent removal of LDL particles from plasma. Gain-of-function mutations of PCSK9 can express as familial hypercholesterolaemia (FH) with high risk of coronary artery disease, whereas PCSK9 deficiency results in low LDL-cholesterol and protection against coronary artery disease [6, 7]. More recently, experimental studies also indicate that PCSK9 could accelerate atherosclerosis by promoting vascular inflammation [8].

High-resolution B-mode carotid ultrasonography has been used for non-invasive detection of subclinical atherosclerosis in large community-based cohorts [9]. We and others have demonstrated that carotid intima-medial wall thickness (IMT), a measure of subclinical atherosclerosis that predicts incident coronary heart disease, is correlated with standard cardiovascular risk factors [10-12]. A report by Cohen et al also found that genetic variations in PCSK9 were associated with changes in carotid IMT in a large population study [7]. In the present study, we investigated the association between serum PCSK9 concentration and carotid IMT in a cross sectional, community-based sample of asymptomatic subjects in Western Australia.

METHODS

Study population

The data presented here were derived from a sample of participants in the Perth Carotid Ultrasound Disease Assessment Study (CUDAS) [12, 13]. The selection criteria and study design of this community-based study have been detailed previously. The participants were randomly selected from the Perth Community population and assessed for cardiovascular risk factors and had carotid B-mode ultrasound performed. This present study sample was confined to the 295 asymptomatic subjects (151 men and 144 women; age 53 ± 13 years [mean ± SD], range 28 to 77) in whom we had available serum (stored at -70^{o}C) to measure PCSK9. A self-administered questionnaire similar to that used by the 1994 Australian National Heart Foundation Perth Risk Factor Prevalence Survey was used to record a history of smoking, hypertension, hyperlipidaemia, diabetes, angina pectoris, myocardial infarction, stroke or a family of premature-onset myocardial infarction (MI) or stroke by age 55 years in first degree relatives. Anthropomorphic measurements and the lower of two resting blood pressures were recorded. Body mass index (BMI) was calculated as weight (kg)/height (m)^2. The study protocol was approved by Sir Charles Gairdner Group Human Research Ethics Committee (Reference No: 2014-086). Written informed consent was obtained from all study participants.

Biochemical analysis

A fasting blood sample was obtained from each subject. The methods for measurements of biochemical analytes were previously described [12,13].

Total cholesterol, high-density lipoprotein (HDL)-cholesterol, triglyceride and glucose levels were determined enzymatically on a Hitachi 747 autoanalyzer (Tokyo, Japan). The LDL-cholesterol was calculated by Friedewald's method; all individuals has fasting triglyceride levels <4.5 mmol/L. Insulin was measured using a two-site immunoenzymometric assay (Tosoh A1A-600 immunoassay analyser, San Francisco). Lipoprotein(a) (Lp(a)) concentration was determined by rate nephelometry (Beckman-Coulter Inc., Fullerton, CA, USA). Insulin resistance was estimated using the HOMA formula: fasting insulin (mU/L) X fasting plasma glucose (mmol/L)/22.5. Serum PCSK9 and plasma interleukin-6 (IL-6) were measured by immunoassay (R & D Systems); the inter-assay coefficients of variation for these methods were <5%. Serum hs-CRP was measured by a microparticle turbidity assay (Hitachi 917, Roche). Monocyte and white cell counts were measured by a Coulter counter.

Carotid ultrasound

Bilateral carotid B-mode ultrasound was performed by two trained sonographers using a 7.5-MHz annular phased-array transducer on an Interspec (Apogee) CX 200 ultrasound machine as previously described [12,13]. The IMT was defined as the distance between the characteristic echoes from the lumen–intima and media–adventitia interfaces on the far wall of the distal common carotid artery measured over a 1 cm segment length. A thorough search of the distal common carotid, carotid bulb, and internal and external carotid arteries was also made to determine the presence of focal plaque. Plaque was defined as a clearly identified area of focal increased thickness (≥1 mm) of the intima–media layer. Three end-diastolic images were analysed from the right and left distal common arteries at a site free of

any discrete plaque and measurements averaged to give the mean IMT. Repeat measurements of randomly selected scans revealed no significant variation in the IMT measurements. The intraobserver coefficient of variability for image acquisition and analysis in our laboratory was 2.9% for sonographer no. 1 and 4.8% for sonographer no. 2. The interobserver coefficient of variability was 5.9%. The mean (±SD) difference in carotid artery IMT between repeat measurements varied from 0.03 ± 0.02 mm for intraobserver variability to 0.05 ± 0.04 mm for interobserver variability.

Statistical analysis

All analyses were performed using SPSS 21 (SPSS, Inc., Chicago). Associations were examined by simple and multivariate linear regression methods. Logarithmic transformation was used where appropriate for continuous variables with skewed distributions. Outcome variable of the association analyses was mean carotid IMT. Partial regression analysis was performed to adjust the use of cholesterol-lowering mediation (yes or no) for the association between serum PCSK9 and mean carotid IMT. We restricted our selection of independent variables for multivariate regression analysis to those are considered as potential risk factors for increased carotid IMT. These include age, male gender, hypertension, obesity, smoking, glucose, lipids, Lp(a), adipocytokines and plasma makers of chronic inflammation. Statistical significance was defined at the 5% level using a two-tailed test.

Results

The anthropometric, clinical and biochemical data of the 295 subjects are summarized in Table 1. The mean age was 53+13 yrs, with nearly equal number of men and women subjects. The prevalence of hypertension, smoking (current), diabetes, obesity and family history of CVD were 29%, 14%, 2.4%, 23% and 20%, respectively. About 6% of individuals (9 men and 10 women) were taking cholesterol-lowering (statin) mediation. Compared with women, men were more likely to have higher BMI (27+4 kg/m^2 vs 26+5 kg/m^2), plasma triglycerides (1.5+0.8 mmol/L vs 1.2+0.7 mmol/L), monocyte count (0.56+0.18 x109/L vs 0.48+0.15 $x10^9$/L), and lower HDL-cholesterol (1.2+0.3 mmol/L vs 1.5+0.4 mmol/L) and adiponectin (9.2+5.1 mg/L vs 15.4+8.2 mg/L) levels ($P<0.01$ for all). Men also had significantly higher mean carotid IMT (0.73+0.15 mm vs 0.68+0.11 mm, $P<0.01$) and were more likely to have carotid focal plaques (33% vs 22%, $P<0.05$) compared with women. The above-mentioned results were consistent with data from the whole cohort participants as we previously reported (data not shown).

Table 2 shows the relationship of serum PCSK9 and mean carotid IMT with clinical and biochemical parameters in the subjects studied. In univariate analysis, serum PCSK9 concentration was positively ($P<0.05$ in all) associated with age (r=0.204), BMI (r=0.149), waist circumference (r=0.139), systolic blood pressures (r=0.116), glucose (r=0.211), insulin (r=0.178), HOMA score (r=0.195), plasma triglyceride (r=0.285), total cholesterol (r=0.241) and LDL-cholesterol concentrations (r=0.172). Serum PCSK9 concentration was also positively associated with mean carotid IMT (r=0.141,

P<0.05) (Figure 1). A similar association between serum PCSK9 concentration and mean carotid IMT was observed for men (r=0.165, P<0.05) and women (r=0.188, P<0.05). The association between serum PCSK9 concentration and mean carotid IMT remained significant after adjusting for cholesterol-lowering therapy (r=0.136, P<0.05). Exclusion of those 19 individuals who were taking cholesterol-lowering mediation from univariate analysis did not alter the abovementioned association with serum PCSK9 (data not shown). Likewise, mean carotid IMT was positively (P<0.05 for all) associated with age (r=0.664), BMI (r=0.168), waist circumference (r=0.285), systolic blood pressures (r=0.473), glucose (r=0.226), plasma triglycerides (r=0.213), total cholesterol (r=0.254), LDL-cholesterol (r=0.256), Lp(a) (r=0.124), interleukin-6 (r=0.168), monocyte count (r=0.126), and inversely with HDL-cholesterol (r=-0.113). Serum PCSK9 concentrations were not correlated (P>0.05 in all) with diastolic blood pressure, HDL-cholesterol, Lp(a), adiponectin, interleukin-6, hs-CRP, white cell and monocyte counts (P>0.05 for all). Also, no significant association was found between insulin, HOMA score, adiponectin, hs-CRP and white cell count with mean carotid IMT (P>0.05 for all).

In a multivariate regression analysis including male gender, hypertension, smoking status, HOMA score, obesity, cholesterol-lowering mediation and serum PCSK9 (Table 3 Model 1), only male gender, hypertension and serum PCSK9 remained significant predictors of mean carotid IMT (*P*<0.05 in all). Replacing hypertension with systolic blood pressure and obesity with BMI as continuous variables did not alter the results (data not shown). Elevated LDL-

cholesterol, but not plasma triglyceride or Lp(a) concentrations, was also a significant predictor of mean carotid IMT when adding into the model as an independent variable (Table 3 Model 2). Male gender, hypertension, serum PCSK9 and LDL-cholesterol remained significant predictors of mean carotid IMT after the inclusion of plasma IL-6 and monocyte count as additional variables (Table 3 Model 3). Serum PCSK9 concentration remained as a significant and independent predictor of mean carotid IMT even when those 19 individuals who were taking cholesterol-lowering mediation were excluded from multivariate analysis (Models 1 to 3). However, serum PCSK9 concentration was no longer an independent predictor of mean carotid IMT when adding age as a variable in the regression models (Models 1 to 3) (data not shown).

DISSCUSSION

Our major finding was that serum PCSK9 concentration was a significant predictor of carotid IMT in asymptomatic individuals from the community. This association was independent of traditional cardiovascular risk factors including gender, hypertension, smoking, obesity, LDL-cholesterol, triglycerides, Lp(a) and plasma markers of inflammation.

Few studies have examined the association between serum PCSK9 concentration and carotid IMT. Lee et al reported, in 126 hypertensive Korean patients, that serum PCSK9 was associated with carotid IMT independent of age, the lipid profile and other traditional CV risk factors [14]. A similar finding was also observed by Huijgen et al in 112 Dutch healthy individuals recruited from the community [15]. We have extended previous reports by examining the association between serum PCSK9 and carotid IMT in a larger community-based population from Western Australia. We also explored its role as an independent marker from traditional markers, including lipid profile, Lp(a) and inflammatory markers.

It is now well recognized that PCSK9 plays a key role in atherogenesis. Its effects on the development of atherosclerosis are mainly mediated via degradation of hepatic *LDLR*, which impairs the catabolism of LDL and subsequently causes hypercholesterolaemia [5]. Consistent with this, we previously reported that plasma PCSK9 concentration was inversely associated with the catabolism of LDL [16]. Our recent data also suggest a regulatory role for PCSK9 on the catabolism of triglyceride-rich lipoproteins

[17]. Consistent with this, we found that plasma PCSK9 concentration was associated with plasma triglyceride, total cholesterol and LDL-cholesterol concentrations. The significant association between plasma PCSK9 concentration and markers of insulin resistance, such as fasting glucose, insulin and HOMA score, implies the potential role of insulin in PCSK9 regulation with animal studies showing that insulin-mediated activation of SREBP-1c (sterol regulatory element-binding protein 1-c) induces the expression of PCSK9 mRNA (18). Our data also show that serum PCSK9 was a significant predictor of carotid IMT, independent of plasma LDL-cholesterol, triglyceride concentrations and other traditional CV risk factors (Table 3, Models 1 and 2). This suggests an additive contribution of PCSK9 to subclinical atherosclerosis in this population. Although neither plasma triglyceride nor lipoprotein(a) were independent predictors of carotid IMT in this study, the cumulative effect of all these atherogenic lipids and lipoproteins is most likely to impact on progression of carotid atherosclerosis over time.

Our earlier reports found that carotid IMT was positively associated with hs-CRP, IL-6, monocyte and white cell counts in the whole CUDAS population [12]. Since PCSK9 overexpression has been shown to regulate genes involved in inflammation [8], the impact of PCSK9 on carotid IMT may involve mechanisms relating to inflammatory pathways. In this study, we also found that plasma IL-6 concentration and monocyte count were associated with cartoid IMT. However, serum hs-CRP and white cell count were not found to be associated with carotid IMT. The lack of significant associations may reflect lack of statistical power in our current sample size. Interestingly, we

found that serum PCSK9 concentration remained a significant predictor after adjustment for IL-6, monocyte count, lipid profile and other traditional CV risk factors (Table 3, Model 3). This suggests that PCSK9 may have a role in early pathogenesis of atherosclerosis. Measurements of markers of endothelial damage (e.g. ICAM-1, VCAM-1 and von Willebrand factor), other adipocytokines (e.g. TNF-α) and other inflammatory makers (monocyte chemoattractant protein-1 and serum amyloid A) may further help to elucidate the association between PCSK9 and carotid IMT.

Contrary to the study by Huijgen et al [15], we found that the significant association between PCSK9 and carotid IMT was attenuated by age. Compared with the former study, our participants were older (41+8 yrs vs 53+13 yrs). It is possible that increasing age overrides the impact of PCSK9 on carotid IMT; this would have diminished the strength of the association between PCSK9 concentration and carotid IMT seen in our participants. However, this requires further investigation.

This study does have limitations. We only employed a surrogate estimate of atherosclerosis, nevertheless carotid IMT is a predictor of future cardiovascular events. The cross-sectional nature of the design does not allow causal inferences to be drawn. Hence, our results should be viewed with caution. However, two recent clinical trials support the role of PCSK9 on atherosclerosis showing about 50% relative reductions in cardiovascular events with PCSK9 inhibition over 12-18 months in a range of patient populations [19, 20]. The use of cholesterol-lowering mediation (statin) is well

known to affect PCSK9 levels. However, only 6% of our subjects were on cholesterol-lowering mediation. Moreover, exclusion of these individuals from statistical analysis did not alter our principal findings. Our assay of PCSK9 did not differentiate intact and the truncated form of PCSK9 (such as furin-cleaved PCSK9). Hence, more specific methods to quantify these may help to clarify their roles contributing to atherosclerosis.

In conclusion, in this cross-sectional study serum levels of PCSK9 were related to subclinical carotid atherosclerosis, independent of traditional risk factors and other inflammatory markers. Whether PCSK9 inhibition improves clinical outcomes remains to be fully demonstrated in long-term clinical endpoint trials. This is being addressed in several ongoing clinical trials to evaluate the effect of PCSK9 monoclonal antibody for the prevention of cardiovascular events in high risk patients.

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Table 1. Clinical, biochemical and lipoprotein kinetic characteristics of the 295 subjects

	Mean ± SD	Range
Age (Yr)	53 ± 13	28-77
Male gender (n, %)	151 (51)	
BMI (kg /m^2)	27 ± 4	17-41
Waist circumference (cm)	86 ± 13	58-122
Systolic blood pressure (mm Hg)	127 ± 18	85-190
Diastolic blood pressure (mm Hg)	79 ± 11	50-114
Hypertension (n, %)	85 (29)	
Current smoking (n, %)	42 (14)	
Diabetes (n, %)	7(2.4)	
Obesity (n, %)	68 (23)	
Family history CHD (n, %)	58 (20)	
Glucose (mmol/L)	5.6 ± 1.4	3.6-22
Insulin (mU/L)	44 ± 32	2.0-197
HOMA score	11 ± 10	0.3-92
Triglycerides (mmol/L)	1.3 ± 0.7	0.4-4.4
Total Cholesterol (mmol/L)	5.5 ± 1.0	3.3-8.6
HDL-cholesterol (mmol/L)	1.3 ± 0.4	0.6-3.1
LDL-cholesterol (mmol/L)	3.6 ± 0.9	1.6-6.4
Lp(a) (mg/dL)	25 ± 32	2-157
PCSK9 (mg/L)	265 ± 84	83-529
Adiponectin (mg/L)	12 ± 7.5	1.1-51
Interleukin-6 (µg/L)	4.0 ± 2.3	1.6-24
hsCRP (mg/L)	3.3 ± 3.8	1.0-26
White cell count (x10^9/L)	6.4 ± 1.6	3.3-13.3
Monocyte (x10^9/L)	0.52 ± 0.17	0.10-1.2
Mean carotid IMT (mm)	0.71± 0.13	0.44-1.13
Max carotid IMT (mm)	0.84 ± 0.16	0.51-1.56
Presence of focal plaque (n, %)	82 (28)	

Table 2. Associations (Pearson correlation coefficients) of serum PCSK9 and mean carotid IMT with clinical and biochemical parameters in the subjects studied

	PCSK9	Mean carotid IMT
Age (yr)	0.204**	0.664**
BMI (kg /m^2)	0.149*	0.168**
Waist circumference (cm)	0.139*	0.285**
Glucose (mmol/L)	0.211**	0.226**
Insulin (mU/L)	0.178**	0.006
HOMA score	0.195*	0.056
Systolic blood pressure (mm Hg)	0.116*	0.473**
Diastolic blood pressure (mm Hg)	0.100	0.273**
Triglycerides (mmol/L)	0.285**	0.213**
Total Cholesterol (mmol/L)	0.241**	0.254**
HDL-cholesterol (mmol/L)	-0.017	-0.113*
LDL-cholesterol (mmol/L)	0.172**	0.256**
Lp(a) (mg/dL)	-0.038	0.124*
Adiponectin (mg/L)	0.024	0.087
Interleukin-6 (µg/L)	0.043	0.169**
hsCRP (mg/L)	0.014	0.076
White cell count (x10^9/L)	-0.035	0.035
Monocyte (x10^9/L)	0.058	0.126*
PCSK9 (mg/L)		0.141*
Mean carotid IMT (mm)	0.141*	
Max carotid IMT (mm)	0.149*	0.966**

Table 3. Multivariate regression model showing gender, hypertension, PCSK9 and LDL-cholesterol as predictors of mean carotid IMT

Predictor variable	Regression coefficient	Standard Error	P-value
Model 1*			
Male gender	0.188	0.015	0.001
Hypertension (yes or no)	0.164	0.017	0.005
PCSK9 (mg/L)	0.148	0.055	0.011
Adjusted R^2=10%, P<0.001			
Model 2*			
Male gender	0.152	0.015	0.007
Hypertension (yes or no)	0.166	0.016	0.006
PCSK9 (mg/L)	0.112	0.050	0.041
LDL-cholesterol (mmol/L)	0.171	0.009	0.004
Adjusted R^2=13%, P<0.001			
Model 3*			
Male gender	0.158	0.015	0.006
Hypertension (yes or no)	0.151	0.017	0.008
PCSK9 (mg/L)	0.119	0.054	0.042
LDL-cholesterol (mmol/L)	0.155	0.009	0.009
Adjusted R^2=14%, P<0.001			

*Additional variables

Model 1: Current smoking (yes or no), HOMA score, obesity (yes or no), cholesterol-lowering medication (yes or no)

Model 2: Current smoking (yes or no), HOMA score, obesity (yes or no), plasma triglyceride and Lp(a) concentration

Model 3: Current smoking (yes or no), HOMA score, obesity (yes or no), plasma triglyceride and Lp(a) concentration, interleukin-6 and monocyte count

Figure 1 Association between serum PCSK9 and mean carotid IMT in the 295 subjects