Research article

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Serum Proprotein Convertase Subtilisin/ Kexin Type 9 Levels are Increased in Patients with Transient Ischemic Attack

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ABSTRACT

Background: Proprotein Convertase Subtilisin/Kexin type 9 (PCSK9) is associated with hypercholesterolemia and atherosclerotic disease while its inhibition reduces cardiovascular risk. There is some evidence that serum PCSK9 concentrations are higher in patients with acute coronary syndromes compared with those with stable coronary artery disease, which has been attributed to a proatherogenic and prothrombotic state. **Objective:** This case-control pilot study investigated potential associations of PCSK9 in patients with transient ischemic attack (TIA). **Methods:** A total of 20 patients with a first-ever atherosclerotic non-cardioembolic TIA and 20 controls of similar age and sex were enrolled. Clinical characteristics, metabolic parameters, including serum PCSK9 within 24 hours from the onset of TIA symptoms were recorded. **Results:** The serum PCSK9 concentration was higher in TIA patients vs. controls (mean values, 248 ng/mL vs. 196 ng/mL, p = 0.02). In patients with TIA, serum PCSK9 correlated with age (r=0.603, p=0.03), history of coronary artery disease (r=0.515, p=0.020) and ABCD^2 score (Age, Blood pressure, Clinical features, symptom Duration, Diabetes - a future stroke prediction tool) (r=0.512, p=0.021). In multivariate analysis, serum PCSK9 was independently associated with higher odds of TIA (1.16 per 10 ng/mL increase, 95% CI 1.01-1.34, p=0.035). **Conclusions:** Our findings indicate that serum PCSK9 levels are independently associated with atherosclerotic TIA and the risk of future stroke. Further investigation is needed to confirm these findings or to assess the use of PCSK9 as a target for early treatment as well as for secondary stroke prevention.

KEY WORDS: *Transient ischemic attack, proprotein convertase subtilisin/kexin type 9, atherosclerosis, stroke prevention, acute coronary syndromes*

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INTRODUCTION

Transient ischemic attack (TIA) is classically defined as a sudden, focal, neurologic deficit of vascular origin with a duration of less than 24 hours confined to an area

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of the brain, or the eye perfused by a specific artery.[1] The current tissue-based definition of TIAs describes them as transient episodes of neurological dysfunction caused by focal brain, spinal cord, or retinal ischemia, which do not lead to an acute infarction.[2] In general, a TIA is a serious brain vasculature event that is associated with an increased risk of future cardiovascular incidents, including stroke, within weeks to months after the index event.[3-6] Patients with TIAs of non-cardioembolic, atherosclerotic etiology often manifest multiple risk factors of cardiovascular disease (CVD).[7] Early identification and modification of these predisposing factors effectively reduces the recurrent risk of stroke.[8,9] It has been recently reported that urgent evaluation and management of patients with TIA reduces the annual risk of major cardiovascular events to 6.2%.[10] Taking into consideration that TIA represents a high CVD risk state, other secondary stroke prevention strategies could be of value.

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a serine protease that plays a major role in the pathophysiology of atherosclerosis and is significantly associated with an increased risk of cardiovascular events.[11] It binds to low-density lipoprotein (LDL) receptors and promotes their degradation thereby leading to fewer LDL-receptors available on the hepatocyte membrane to remove LDL cholesterol from the circulation.[12,13] Increased plasma levels of PSCK9 have been reported in patients with acute coronary syndromes.[14] Furthermore, there is evidence that inhibition of PCSK9 reduces CVD risk in patients with vascular disease, including stroke.[15] However, there is no evidence regarding the association of PCSK9 with TIA; would confirmatory relevant evidence suggest that PCSK9 might serve as a novel target to reduce CVD risk in TIA patients?

We undertook this case-control pilot study to investigate the associations of PCSK9 with atherosclerotic TIA.

PARTICIPANTS AND METHODS

Study Population

This was a case-control pilot study involving adult patients with first-ever TIA of atherosclerotic, non-cardioembolic etiology. Patients (n=20) were consecutively recruited from May 2017 to December 2017 in the Internal Medicine Departments of the University Hospital of Ioannina and the General Hospital of Ioannina "Georgiou Hatzikosta". The control group comprised of 20 consecutive CVD-free subjects of similar sex and age attending the outpatient setting of both hospitals for routine clinical and laboratory examinations during the same period.

The diagnosis of TIA was made on the grounds of an acute focal neurological deficit (kinetic or sensory, retinal ischemia or dysarthria) with complete resolution within 24 hours and the absence of a recent ischemic legion on brain imaging that justified the acute focal neurological deficit.[16] A diagnostic brain imaging with an urgent cranial non-contrast CT scan was performed – immediately or within an hour upon arrival at the hospital - to exclude stroke mimics and hemorrhage. A follow-up CT or an MRI scan was performed after 24-48 hours. A cardiological work up was offered to TIA patients to exclude cardiac conditions that could be a source of an embolus (including atrial fibrillation, severe heart failure, infectious or non-infectious endocarditis, left ventricular myxoma). Neurological and hematological evaluation was enlisted to exclude patients with stroke mimics, and/or hematological conditions, such as polycythemia, sickle cell anemia, coagulation disorders, antiphospholipid syndrome, and severe anemia. Subjects with clinical suspicion or laboratory evidence of acute or chronic infection or inflammatory condition were excluded from the study.

Demographic and clinical characteristics were recorded in all eligible patients and controls: age, sex, body mass index (BMI, kg/m^2), smoking habits (i.e. smoking within the past 3 months), hypertension (history of hypertension and/or antihypertensive treatment) dyslipidemia (history and or lipid-lowering treatment), diabetes mellitus (history and or antidiabetic treatment), history of coronary artery disease (CAD) or peripheral artery disease (PAD).

The ABCD^2 score (age, blood pressure, clinical findings, duration of symptoms, and presence or absence of diabetes), the modified Rankin scale (mRS) and the National Institutes of Health Stroke Scale (NIHSS) were recorded upon admission. Patients were followed up for a week, a month and six months after admission.

All participants gave informed consent and the protocol was approved by the institutional ethics committee.

Laboratory measurements

Blood samples, after at least 12 hours of fasting and within 24 hours from the onset of TIA symptoms, were collected for the determination of metabolic parameters including the lipid profile. The glomerularfiltration rate (eGFR) was calculated using the chronic kidney disease epidemiology collaboration (CKD-EPI) equation.[17]

PCSK9 determination

The serum PCSK9 concentrations were measured by a high-sensitivity, quantitative sandwich enzyme immunoassay (Quantikine ELISA, R & D Systems Europe Ltd) with a mean detectable concentration of 0.096 ng/mL.[18] Upon the day of the assay standard solutions and serum samples were pipetted into the pre-coated with a mono-

clonal antibody against human PCSK9 wells of the plate. Therefore, any PCSK9 present in the solutions was bound by the pre-coated antibody. All the unbound material was washed away and an enzyme-linked polyclonal antibody specific for the human PCSK9 was added to the wells. At that point a substrate solution was added to the wells. Color developed according the concentration of the PCSK9 serum samples and the standard solutions. A microplate reader was used to determine the optical absorbance for each well of the plate at 450 nm.

Statistical analysis

Categorical variables are presented as frequencies and percentages. Continuous variables are presented as means ± standard deviation (if normally distributed) or median (range) in case of non-normally distributed variables. The Kolmogorov–Smirnov test was used to analyze normality. Comparisons of clinical characteristics and biochemical parameters between groups were assessed by using $\chi 2$-(chi-squared) test for categorical variables, Student's t-test (parametric variables) for independent samples and Mann-Whitney U test (non-parametric variables). The bivariate Pearson correlation was used to measure the strength and direction of the linear relationships between PCSK9 concentrations and clinical and laboratory parameters in patients and controls. The associations with TIA with clinical and biochemical parameters were evaluated by binary logistic regression analysis: for the univariate analysis, the level of significance was set at 10% to reduce the risk of a type II error, while in the final multivariate analysis the level of significance was set at 5%.

Two-tailed tests were used to determine significance at the 5% level. Statistical analyses were performed by the SPSS software for Microsoft windows (SPSS Inc, version 25.0 for Windows; Chicago, IL).

RESULTS

Baseline characteristics

The demographics and clinical characteristics of the study population are shown in Table 1. There were no significant differences between patients and controls with regard to age, sex, BMI, smoking habits, history of hypertension, dyslipidemia, diabetes or baseline drug therapy.

No differences in metabolic parameters in TIA vs. control were shown (Table 2). Of note, serum PCSK9 concentration was higher in TIA patients (mean values, 248 ng/mL vs. 196 ng/mL, p = 0.02) (Table 2). In patients with a TIA, the serum PCSK9 concentration correlated with the age (r=0.603, p=0.03), the history of coronary artery disease (CAD) (r=0.515, p=0.020) and the $ABCD^2$ score (r=0.512, p=0.021) (Table 2). In the control group serum PCSK9 concentrations correlated with the history of dyslipidemia (r=0.027, p=0.027), and HDL cholesterol levels (r=0.474, p=0.035).

In univariate binary logistic analysis, serum PCSK9 (odds ratio, OR, per 10 ng/mL increase, 1.17, 95% Confidence Interval, CI 1.02-1.34, p = 0.028) and HDL-cholesterol concentrations (OR 1.06, 95% CI 0.99-1.12, p =0.089) were

TABLE 1. Demographic and clinical characteristics of the study population on admission.

Variable	TIA patients (N=20)	Control group (N=20)	p-value
Age, years	68±13	68±9	0.946
Men, n (%)	14 (70)	16 (80)	0.782
BMI, kg/m^2	27.8±2.6	29.2±4.7	0.394
Smoking, n (%)	6 (30)	5 (25)	0.789
Hypertension, n (%)	10 (50)	10 (50)	N/A
Dyslipidemia, n (%)	11 (55)	11 (55)	N/A
Diabetes, n (%)	6 (30)	6 (30)	N/A
CAD, n (%)	2 (10)	0 (0)	N/A
$ABCD^2$ score	3.85±1.46	N/A	N/A
mRS	2.85±1.18	N/A	N/A
NIHSS	5.05±2.46	N/A	N/A
Drug therapy	**n (%)**	**n (%)**	
Antihypertensives	10 (50)	10 (50)	N/A
Oral hypoglycemic therapy	6 (30)	6 (30)	N/A
Statin	10 (50)	11 (55)	N/A
Aspirin	3 (15)	2 (10)	0.673
Biochemical data			
Total-cholesterol, mg/dL	191±42	177±38	0.267
LDL-cholesterol, mg/dL	110±35	103±29	0.626
Triglycerides, mg/dL	135±64	134±58	0.914
HDL-cholesterol, mg/dL	53±15	46±7	0.126
Apolipoprotein B, mg/dL	87±26	82±23	0.665
Apolipoprotein A1, mg/dL	139±22	152±21	0.088
Lipoprotein(a), mg/dL	12 (2-66)	8 (2-132)	0.343
PCSK9, ng/mL	248±94	196±29	0.020

Abbreviations: N/A, non-applicable; BMI, body mass index; CAD, coronary artery disease; mRS, modified Rankin scale; NIHSS, National Institutes of Health Stroke Scale.
Conversions: To convert total cholesterol, LDL-C or HDL-C from [mg/dl] to [mmol/L] multiply by 0.02586; to convert TG from [mg/dL] to [mmol/L] multiply by 0.0113.

TABLE 2. Correlation of clinical and laboratory parameters with PCSK9 in the population.

Variable	TIA patients		Control group	
	r	P-value	r	P-value
Age, years	0.634	0.003	-0.380	0.099
Male sex	-0.057	0.810	-0.075	0.752
BMI, kg/m^2	0.562	0.438	-0.259	0.271
Smoking	-0.216	0.360	0.060	0.800
Hx of hypertension	0.033	0.890	-0.036	0.881
Hx of dyslipidemia	0.393	0.086	0.493	0.027
HX of diabetes mellitus	0.515	0.020		
Hx of CAD			N/A	N/A
Hx of PAD	N/A	N/A	N/A	N/A
Systolic BP	-0.196	0.408	0.191	0.419
Diastolic BP	-0.416	0.068	0.158	0.505
TC, mg/dL	0.066	0.781	0.188	0.427
TG, mg/dL	0.061	0.797	0.003	0.991
HDL-C, mg/dL	0.387	0.092	0.474	0.035
LDL-C, mg/dL	-0.107	0.653	0.142	0.550
ApoA1, mg/dL	0.299	0.200	0.322	0.166
ApoB, mg/dL	-0.291	0.213	0.184	0.437
Lp (a), mg/dL	-0.325	0.162	0.091	0.703
ABCD2	0.512	0.021	N/A	N/A
mRS	-0.116	0.625	N/A	N/A
NIHSS	-0.016	0.947	N/A	N/A

Abbreviations: Hx: history; N/A: non-applicable; BMI: body mass index; CAD: coronary artery disease; PAD: peripheral arterial disease; BP: blood pressure; The ABCD2 score is based on five parameters (age, blood pressure, clinical features, duration of TIA, and presence of diabetes); mRS: modified Rankin scale; NIHSS: National Institutes of Health Stroke Scale; TC: total cholesterol; LDL-C: low-density lipoprotein cholesterol; TG: triglycerides; HDL-C: high-density lipoprotein cholesterol; Apo: apolipoprotein; Lp: lipoprotein; PCSK9: proprotein convertase subtilisin/kexin type 9.
Conversions: To convert total cholesterol, LDL-C or HDL-C from [mg/dl] to [mmol/L] multiply by 0.02586; to convert TG from [mg/dL] to [mmol/L] multiply by 0.0113;

associated with higher odds of TIA (Table 3). In multivariate analysis, only serum PCSK9 was independently associated with TIA (OR per 10 ng/mL increase, 1.16, 95% CI 1.01-1.34, p = 0.035) (Table 3).

DISCUSSION

Our study suggests that serum PCSK9 levels are independently associated with higher odds of TIA and correlate with the risk of future stroke in TIA patients as estimated by the ABCD^2 score. Although this was a small pilot study, intriguing findings emerged. Our study confirmed previously reported associations of serum PCSK9 concentrations with increasing age,[19] but failed to show any correlations with other clinical characteristics (e.g. sex, obesity), metabolic parameters or lipidemic profile.

The role of PCSK9 with regard to hypercholesterolemia and atherosclerosis is well studied. Gain of function mutations in PCSK9 cause hypercholesterolemia, while heterozygous nonsense mutations in PCSK9 results in significantly lower plasma LDL cholesterol levels[20, 21] The mechanism that explains these findings is the degradation of the LDL receptors by PCSK9. The extra membrane portion of the LDL receptors have a binding site (EGF-A, Epidermal Growth Factor A like domain) for PCSK9; when PCSK9 and the LDL particle are bound on the LDL receptor, the resulting complex is internalized into an endosome,[22] in a clathrin mediated phagocytosis process that ends up to the degradation of the LDL receptor.[23] Classically, the association of PCSK9 with atherosclerosis has been linked to fewer available LDL receptors on the hepatocyte membrane surface which leads to increased circulating LDL 'proatherogenic' particles.

There is evidence that increased serum PCSK9 levels are associated with severe atherosclerosis and higher rates of acute cardiovascular events, triggered by unstable coronary plaques[24]. Two retrospective, angiographic studies reported an increase in PCSK9 levels in patients with acute myocardial infarction versus stable CAD.[14] PCSK9 is linked to plaque vulnerability through several pathways, including proinflammatory LDL oxidation and direct modification of plaque composition.[25] Of interest, it has also been reported that PCSK9 is involved in platelet activation at the site of a ruptured plaque. Indeed, PCSK9 levels have been shown to correlate with the concentration of thromboxane 11-dh-TXB2 in the urine of patients with acute coronary syndrome, thus suggesting that PCSK9 might be implicated in promoting platelet activation.[26] Besides LDL receptor, PCSK9 can also bind to the apolipoprotein E receptor-2 (ApoER2) which has a 46% homology in its sequence with the LDL receptor. This binding activates cytosolic phospholipase A2 (cPLA2) through the p38MAPK pathway[27]. The cascade of platelet activation is then activated with the release of arachidonic acid. A direct association between PCSK9 serum levels and residual platelet reactivity has been demonstrated in patients with acute coronary syndromes (ACSs) treated with P2Y12 inhibitors. Recombinant human PCSK9 added in vitro to human platelets has been shown to potentiate their activation. Finally, blood clotting factor VIII (FVIII), which has been associated with increased risk of stroke and ACS, is cleared from the circulation by members of

TABLE 3. Binary logistic regression analysis for the associations of biochemical parameters with TIA (selected demographic, clinical and laboratory parameters are shown).

Variable	Univariate analysis			Multivariate analysis		
	Odds ratio	95% CI	P-value	Odds ratio	95% CI	P-value
Age, years	0.99	0.94-1.06	0.879			
Male sex	1.71	0.40-7.34	0.468			
BMI, kg/m^2	0.93	0.72-1.19	0.557			
Hx of hypertension	1.00	0.29-3.45	1.000			
TC	1.01	0.99-1.03	0.283			
TG	1.00	0.99-1.01	0.967			
HDL-C, mg/dL	1.06	0.99-1.12	0.089	1.05	0.97-1.13	0.20
LDL-C, mg/dL	1.01	0.99-1.03	0.512			
ApoA1, mg/dL	0.97	0.94-1.00	0.088			
ApoB, mg/dL	1.01	0.98-1.03	0.535			
Lp (a), mg/dL	0.99	0.97-1.01	0.618			
PCSK9 (per 10 ng/mL increase)	1.17	1.02-1.34	0.028	1.16	1.01-1.34	0.035

Abbreviations: Hx: history; BMI: body mass index; TC: total cholesterol; LDL-C: low-density lipoprotein cholesterol; TG: triglycerides; HDL-C: high-density lipoprotein cholesterol; Apo: apolipoprotein; Lp: lipoprotein; PCSK9: proprotein convertase subtilisin/kexin type 9.
Conversions: To convert total cholesterol, LDL-C or HDL-C from [mg/dl] to [mmol/L] multiply by 0.02586; to convert TG from [mg/dL] to [mmol/L] multiply by 0.0113;

the LDL receptor family. Given that PCSK9 degrades LDL receptors, it is possible that PCSK9 inhibitors by enhancing the expression of LDL receptors may decrease circulating FVIII.[28] Overall, the involvement of PCSK9 in plaque vulnerability and platelet activation represent "attractive" underlying pathophysiologic mechanisms for an increased risk of TIA in subjects with elevated PCSK9.

Our findings should be interpreted in the light of certain limitations. This is a small pilot study with a cross-sectional design. Selection bias could not be completely obviated in spite of the applicability of strict inclusion criteria and methodology. Results of binary regression analysis suggest the involvement of PCSK9 in development of TIA. However, although small sample size is a limiting factor for multivariate binary regression analysis, these findings should be further evaluated by multivariate logistic regression analysis with parameters which correlate with PCSK9 as confounders. On the other hand, in animal studies ischemia itself triggers upregulation of PCSK9 synthesis, which leads to an increase of serum PCSK9 concentration 12 to 96 hours (peak 48 hours) after an acute myocardial infarction .[29] In this respect, a study of PCSK9 kinetics in both the acute and post-acute (stable) state of patients with TIA may be warranted. Of note, in a study including a total of unselected 650 patients admitted with stroke or TIA to a hyper-acute stroke unit it was estimated that the percentage of patients suitable for the newly available cholesterol-lowering treatments was up to 13%.[30]

In conclusion, this pilot study provides intriguing evidence connecting serum PCSK9 with TIA and the risk of future stroke. By putative analogy with acute coronary syndromes, treatment with PCSK9 inhibitors could benefit patients with TIA through reduction of LDL cholesterol levels and through early plaque stabilization via anti-inflammatory and antithrombotic mechanisms. Therefore, to confirm these findings or to evaluate PCSK9 as a treatment target in the setting of TIA needs further investigation.

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ΠΕΡΙΛΗΨΗ

Η Συγκέντρωση της Proprotein Convertase Subtilisin/Kexin Type 9 (PCSK9) είναι υψηλή στον ορό ασθενών με παροδικό ισχαιμικό αγγειακό εγκεφαλικό επεισόδιο (TIA)

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Εισαγωγή: Ο συγκεντρώσεις στον ορό της πρωτεϊνικής κονβερτάσης σουμπτιλισίνη /κεξίνη τύπου 9 (PCSK9) σχετίζονται με υπερχοληστερολαιμία και νοσήματα που οφείλονται σε αθηροσκλήρωση, ενώ είναι γνωστό ότι η αναστολή της μειώνει τον καρδιαγγειακό κίνδυνο. Η συγκέντρωση της PCSK9 στον ορό είναι αυξημένη σε ασθενείς με οξύ στεφανιαίο σύνδρομο λόγω της προαθηρογόνου και προθρομβωτικής κατάστασης. **Σκοπός:** Αυτή η μελέτη ελέγχου ασθενών μαρτύρων διερεύνησε τις συσχετίσεις των συγκεντρώσεων της PCSK9 στον ορό ασθενών με παροδικό ισχαιμικό εγκεφαλικό επεισόδιο (TIA) με τις κλινικοεργαστηριακές παραμέτρους. **Υλικό - Μέθοδοι:** Στη μελέτη συμπεριλήφθηκαν συνολικά 20 ασθενείς με πρώτο επεισόδιο αθηροσκληρωτικής, μη καρδιοεμβολικής, αιτιολογίας TIA, και 20 μάρτυρες παρόμοιας ηλικίας και φύλου. Κατεγράφησαν τα κλινικά χαρακτηριστικά, οι μεταβολικές παράμετροι, συμπεριλαμβανομένων των τιμών της συγκέντρωσης της PCSK9 στον ορό εντός 24 ωρών από την έναρξη των συμπτωμάτων του TIA. **Αποτελέσματα:** Η συγκέντρωση της PCSK9 στον ορό ήταν υψηλότερη στους ασθενείς με TIA έναντι των μαρτύρων (μέσες τιμές, 248 ng/mL έναντι 196 ng/mL, p=0,02). Σε ασθενείς με TIA, η συγκέντρωση της PCSK9 στον ορό συσχετίστηκε με την ηλικία (r=0,603, p=0,03), το ιστορικό στεφανιαίας νόσου (CAD) (r=0,515, p=0,020) και τη βαθμολογία ABCD2 (r=0,512, p=0,021). Με πολυπαραγοντική ανάλυση, η συγκέντρωση της PCSK9 στον ορό συσχετίστηκε ανεξάρτητα με υψηλότερη πιθανότητα για TIA (Odds 1,16 ανά αύξηση 10 ng/mL, 95% CI 1,01-1, 34, p=0,035). **Συμπεράσματα:** Τα ευρήματά μας δείχνουν ότι οι συγκεντρώσεις της PCSK9 στον ορό σχετίζονται ανεξάρτητα με τα αθηροσκληρωτικής αιτιολογίας TIA και τον κίνδυνο μελλοντικού εγκεφαλικού επεισοδίου. Για να επιβεβαιωθούν αυτά τα ευρήματα ή για να αποτελέσει η PCSK9 στόχο στη θεραπεία για την πρόληψη του εγκεφαλικού επεισοδίου ή την έγκαιρη θεραπεία του απαιτείται περαιτέρω διερεύνηση.

ΛΕΞΕΙΣ ΚΛΕΙΔΙΑ: *Παροδικό ισχαιμικό αγγειακό εγκεφαλικό επεισόδιο, proprotein convertqse subtilisin/kexin type 9, αθηροσκλήρωση, πρόληψη εγκεφαλικών, οξύ στεφανιαίο σύνδρομο*

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