The link between proprotein convertase subtilisin/kexin 9 serum levels and E670G gene polymorphism and the risk of ischemic stroke in patients of the south of Egypt

Ghada M. Ezzat[a], Ahmed Nasreldein[b], Ghada A. Mohamed[c], Wael Abdelgwad Elsewify[d], Marwa A. Dahpy[a]

[a]Department of Medical Biochemistry and Molecular Biology, Faculty of Medicine, [b]Department of Neurology and Psychiatry, Faculty of Medicine, [c]Department of Internal Medicine, Faculty of Medicine, Assiut University, Assiut, [d]Department of Internal Medicine, Faculty of Medicine, Aswan University, Aswan, Egypt

Correspondence to Ahmed Nasreldein, Lecturer of Neurology and Psychiatry, PhD, Doctor Degree of Neurology and Psychiatry, Neurology and Psychiatry Department, Faculty of Medicine, Assiut University, Assiut
e-mail: d_ahmednasr@yahoo.com.
Marwa A Dahpy,
Lecturer of Medical Biochemistry and Molecular Biology, PhD, Doctorate Degree of Medical Biochemistry and Molecular Biology, Faculty of Medicine, Assiut University, Assiut, Egypt.
Code: 71515;
https://orcid.org/0000-0002-6007-5691,
e-mail: marwadahpy@yahoo.com
marwadahpy@aun.edu.eg;
Fax: 088-2080278

Received 16 April 2020
Revised 20 August 2020
Accepted 29 September 2020
Published 11 June 2021

Journal of Current Medical Research and Practice
2021, 6:200–207

Background
Proprotein convertase subtilisin/kexin 9 (PCSK9) has a role in low-density lipoprotein-cholesterol metabolism, which relates to the initiation and progression of atherosclerosis and hence cerebrovascular ischemic stroke (CIS). This hospital-based study aims to investigate whether PCSK9 serum levels and *E670G* single nucleotide polymorphism (SNP) of the PCSK9 gene associated with a higher incidence of atherosclerosis and CIS among a patient series in the south of Egypt or not.

Materials and methods
A total of 100 patients with ischemic stroke (68 with atherosclerotic ischemic stroke and 32 nonatherosclerotic ischemic stroke), together with 100 age-matched and sex-matched healthy controls, were enrolled in this study. Genotyping of *E670G* SNP was performed by restriction fragment length polymorphism PCR. The serum PCSK9 levels were determined using ELISA kit, whereas serum lipid profiles were determined using colorimetric methods.

Results
Higher serum PCSK9 levels were significantly noticed in atherosclerotic ischemic stroke. Genotyping distribution of *E670G* gene polymorphism of *PCSK9* gene showed higher distribution in both patients with ischemic stroke and those with atherosclerotic ischemic stroke. The GA heterozygous carriers were at risk of developing ischemic stroke (odds ratio = 2.234, confidence interval = 1.2323–4.0509, P = 0.0081) when compared with controls. Carriers of G allele of *E670G* SNP had significantly higher serum total cholesterol and low-density lipoprotein-cholesterol levels in atherosclerotic ischemic stroke subgroup.

Conclusion
Serum PCSK9 levels were significantly higher among patients with atherosclerotic CIS. The gain-of-function mutation in carriers of the G allele of *E670G* SNP plays an essential role in the pathogenesis of lipid related diseases, and it affects atherogenesis. These findings make the G allele of *E670G* SNP of *PCSK9* gene a possible genetic risk factor for ischemic stroke among atherosclerotic patients.

Keywords:
atherosclerosis, E670G, ischemic stroke, proprotein convertase subtilisin/kexin 9, restriction fragment length polymorphism PCR

J Curr Med Res Pract 6:200–207

2357-0121

Introduction

Ischemic stroke is a major cause of death and disability throughout the world [1]. The epidemiological study of stroke and its risk factors done in Assiut governorate, Egypt, showed that the crude prevalence rate of ischemic stroke was 895/100 000 [2]. Dyslipidemia is considered the most common risk factor for stroke [3]. The accumulation of oxidized low-density lipoprotein-cholesterol (LDL-C) in the vascular endothelium leads to injury and atherosclerosis of the cerebrovascular system [4]. Single nucleotide polymorphisms (SNPs) of genes involved in lipid metabolism regulation are associated with the occurrence of ischemic stroke [5].

Proprotein convertase subtilisin kexin 9 (PCSK9) is a serine protease; its gene is located on the small arm of chromosome 1p32.3 and comprises 12 exons and 11 introns [6]. It is highly polymorphic, with a total of 163 mutations identified so far. Circulating PCSK9 regulates LDL-C plasma levels. It acts as a molecular chaperone that effectively binds to the epidermal growth factor-like domain A of LDL receptor (LDLR) locating on the surface of hepatocytes surface, which mediates approximately 70% of LDL-C clearance, through the promotion of the intracellular lysosomal degradation of hepatic

LDLR[7] and enhancing controlled post-translational targeted degradation, thus resulting in fewer surface LDLRs and higher plasma cholesterol levels [8], leading to a significant elevation in plasma LDL-C levels [9–12]. Besides the liver, PCSK9 is expressed in the small intestine, kidney, and brain; it also binds to the LDL receptor-related protein 1 (LRP1) and the scavenger type B cells. Cell lines, animal models, and genetic studies have revealed the role of PCSK9 in several CNS diseases, including Alzheimer's disease, alcohol use disorder, ischemic stroke, and neuropsychiatric disorders [8].

Proprotein convertase subtilisin kexin 9 *(PCSK9) E670G*NC_000001.10:g. 55529187 G>A SNP is associated with increased concentrations of LDL-C through reduction of LDLR expression on the cell surface, which inhibits cellular uptake of serum LDL-C and ultimately elevates serum cholesterol, which may lead to autosomal dominant hypercholesterolemia and premature atherosclerosis [6].

The results of previous studies about the influence of *E670G SNP* of *PCSK9 gene* on LDL-C level and ischemic stroke were inconclusive [13–16]. Pooled effects of three meta-analyses between 2015 and 2017[17] indicated that G allele carriers had a higher risk of coronary artery diseases and LDL-C levels than noncarrier.

There are available data about the beneficial use of a PCSK9 inhibitor in Egyptian patients with atherosclerosis [18]; however, no previous studies have been conducted to inspect the association between PCSK 9 serum level and E670G SNP of *PCSK9* gene and the risk of ischemic stroke in Egypt.

This study aimed to investigate this role of PCSK9 in patients with ischemic stroke and to determine the distribution and the associated effects of *E670G* SNP of*PCSK9* gene with the risk of ischemic stroke in a hospital-based sample of upper Egyptian patients.

Materials and methods

Our case–control study was conducted during the period from June 2017 to June 2018. A total of 100 patients with ischemic stroke were recruited from inpatients ward in Neurology Department in cooperation with Medical Biochemistry Department and Internal Medicine Department of the Faculty of Medicine, Assiut University, and Internal Medicine Department, Aswan University, Egypt, along with 100 age-matched and sex-matched completely healthy control volunteers without a history of ischemic stroke.

The study was approved by ethics committee of faculty of Medicine, Assiut University Approval number is IRP No: 17300407. Written consent was provided by all participants at the start of the study.

Inclusion criteria

The selected patients fulfilled the following inclusion criteria: age greater than 18 years and acute ischemic stroke presenting within 48 h diagnosed according to WHO diagnostic criteria. Stroke severity was assessed on admission (quantified with the National Institute of Health Stroke Scale). Ischemic stroke group was subdivided into two subgroups: patients with atherosclerotic ischemic stroke (n = 68) and patients with nonatherosclerotic ischemic stroke (n = 32). Patients with nonischemic causes of stroke and those refused to participate were excluded from the study.

Exclusion criteria included previous hemorrhagic stroke, severe heart failure, severe renal failure, malignancy within the past years, and any other severe concomitant noncardiovascular disease.

Detailed medical and neurological history and examinations were performed for all patients. Computed tomography brain was done for all patients to confirm the diagnosis of acute ischemic stroke, and some patients with normal computed tomography brain had additional MRI of the brain to confirm the diagnosis.

Trial of Org 10172 in Acute Stroke Treatment (TOAST), is a system for categorization of ischemic stroke subtypes that mainly based on the etiology of CIS was used.

The TOAST classification includes five subtypes of ischemic stroke: 1) large-artery atherosclerosis, 2) cardioembolism, 3) small-vessel occlusion, 4) stroke of other determined etiology, and 5) stroke of undetermined etiology.

All patients had ECG and echocardiography. A complete extracranial and intracranial ultrasound assessment, with a color-coded duplex ultrasound for the cervical arteries and 2–5-MHz phased array probe for the intracranial arteries, was performed. The extracranial vessels were examined using an examination protocol, and atherosclerotic changes were interpreted according to the criteria published by the Society of Radiologists in Ultrasound [19].

All patients signed an informed consent before they were enrolled in our study.

The study was approved by the Institutional Review Board of Faculty of Medicine/Assiut University (IRP No: 17300407).

Laboratory workup was done for all patients. A volume of 5 ml of venous blood was taken after overnight fasting from all the patients and controls. Of the 5 ml, 1 ml of blood was poured into EDTA-containing tubes and used for DNA extraction, and the remaining 4 ml of blood was used for serum separation by centrifugation at 3000 rpm. Samples were kept at −70°C until the biochemical assays. Serum levels of PCSK9 were measured by ELISA kit supplied by Elabscience ChinaBiotechnology Inc. (Elabscience Company, China catalog number, E-EL-H1579). The serum levels of total cholesterol (TC), triglyceride (TG), and high-density lipoprotein-cholesterol were measured by colorimetric method by kits supplied by Biodiagnostics (Egypt) (catalog no. CH1220, TR2030, and CH1232, respectively) (Biodiagnositics, EGYPT),(Qiagen,USA),(Jena Bioscience,Germeny). Serum concentrations of LDL-C were calculated according to Friedewald equation [20]. DNA was extracted from the peripheral whole blood sample by QIAamp DNA mini extraction kits, catalog no. 51104 supplied by Qiagen (Germany). The DNA was kept at – 20°C until genotyping. Amplification of *E670G* polymorphism of *PCSK9* gene was done through 50-µl PCR reaction using 25-µl thermo scientific dream taq green PCR master mix (2X), 1 µmol/l of each primer, and 1 µg template DNA. The PCR cycling conditions were as follows: initial denaturation for 3 min at 95°C, denaturation at 95°C, annealing at 55°C, and extension at 72°C, repeated for 30 cycles that last for 30 Sec.

Restriction fragment length polymorphism for *E670G SNP* of PCSK9 gene was done by MnI1restriction enzyme (Jena Bioscience, Germany).

Digestion was done at 37°C for overnight using 1 µg PCR product (169 bp), 5 µl of 10 × universal buffer, 10 units of restriction enzyme, and up to 30 µl of PCR-grade water.

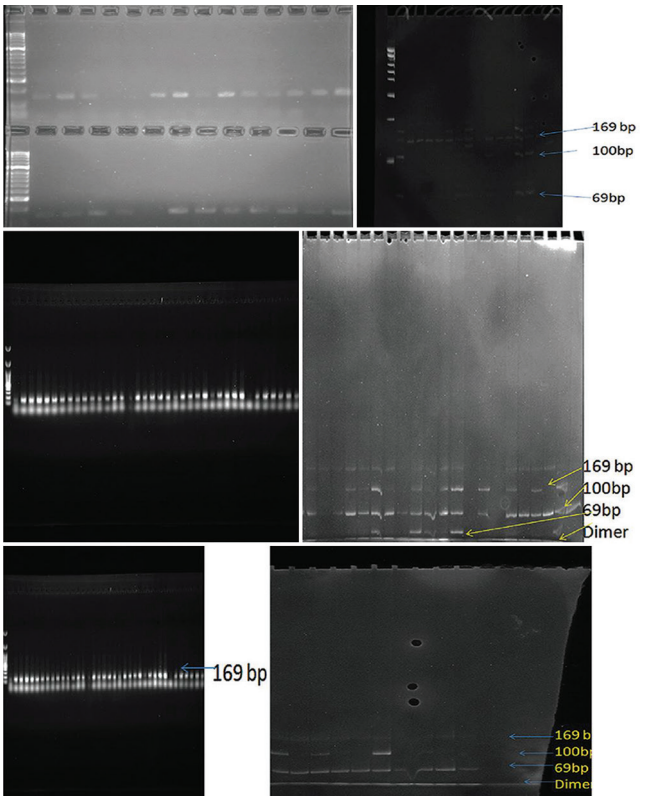
After digestion, the product was visualized by electrophoresis on 10% polyacrylamide gel. To verify genotyping quality, the genotyping call was carried out by two independent personnel, and 25 random samples were re-genotyped, and both showed 100% concordance.

The primers used for detection of *E670G* SNP of *PCSK9* gene included forward primer: 5′-TACGCCGTAGACAACACG-3′, and reverse primer: 5′-TCCCCA GACACCCATCCTGG-3′ [21].

Statistical analysis

The statistical analyses were performed using SPSS 20 (SPSS Inc., Chicago, Illinois, USA). Data were normally distributed, so results were expressed as mean ± SD. Categorical data, genotypes, and alleles were presented by frequencies and percentage. Unpaired sample *t*-test was used for the analysis of the difference between two groups, and analysis of variance test followed by post-hoc test with least significant difference was used for multiple comparisons between different variables. The differences in the frequencies of hypertension, atherosclerosis, alleles, and genotypes were analyzed using Chi-square test. Hardy-Weinberg equilibrium (HWE) was assessed by χ^2 analysis. The odds ratio (OR) and 95% confidence interval (CI) were calculated by Medcalc software (*MedCalc statistical software on line: https://www.medcalc.org/*). Pearson's correlation was used to evaluate the association between different parameters. The *P* values were significant if less than 0.05.

Figure 1



10% polyacrylamide electrophoresis image showing PCSK9 RFLP-PCR products amplified from blood genomic DNA for the detection of PCSK9 rs505151 gene polymorphism. Left: whole gene (169 bp) detected before digestion by MnI1 restriction enzyme against 3000 bp ladder. Right: genotyping after digestion by MnI1 restriction enzyme. The AG genotype is identified by the presence of 3 fragments of 169, 100, and 69.The AA genotype is identified by two fragments of 100 and 69 bp, whereas the GG genotype is identified by the presence of one fragment of 169 bp (not demonstrated).

Table 1 Demographic data and biochemical parameters of ischemic stroke group in comparison with controls

Laboratory data	Control group (*n*=100) (mean±SD)	Ischemic stroke group (*n*=100) (mean±SD)	*P*
Male [*n* (%)]	60 (60.0)	66 (66.0)	0.380
Female [*n* (%)]	40 (40.0)	34 (34.0)	
Age (years)	64.40±10.29	64.86±10.19	0.751
Age (years) (range)	40-85	40-85	
PCSK9 (ng/ml)	42.90±7.73	79.40±10.30	<0.001**
TC (mg/dl)	152.10±40.19	328.46±84.94	<0.001**
HDL-C (mg/dl)	42.66±15.88	46.64±17.23	0.0910
LDL-C (mg/dl)	101.10±43.05	234.76±82.32	<0.001**
TG (mg/dl)	119.31±41.36	242.99±78.78	<0.001**
VLDL (mg/dl)	9.80±6.36	47.07±16.63	<0.001**

Sample *t*-independent test. HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; PCSK9, proprotein convertase subtilisin/kexin 9; TC, total cholesterol; TG, triglyceride; VLDL, very low-density lipoprotein cholesterol. *P*>0.05 (NS). **P*<0.05 (significant). ***P*<0.001 (highly significant), P value between cases and controls.

Table 2 Genotyping and allelic frequencies distribution of E670G SNP of *PCSK9* gene in controls and CIS group

Genotype	Control group (*n*=100) [*n* (%)]	CIS cases group (*n*=100) [*n* (%)]	OR	95% CI	*P*
AA*	68 (68.0)	50 (50.0)	Reference	Reference	Reference
AG	28 (28.0)	46 (46.0)	2.234	**1.232-4.0509**	0.008
GG	4 (4.0)	4 (4.0)	1.3600	0.3244-5.7014	0.674
Recessive					
AA + AG	96 (96)	96 (96)	1.0000	0.2430-4.1145	1.0000
GG	4 (4)	4 (4)			
Dominant					
AA	68 (68)	50 (50)	2.1250	**1.1962-3.7750**	0.010
AG + GG	32 (32)	50 (50)			
Additive AA	68 (68)	50 (50)	1.36	0.3244-5.7014	0.674
GG	4 (4)	4 (4)			
Alleles A*	164 (82)	146 (73)	1.6849	**1.0557-2.7150**	0.032
G	36 (18)	54 (27)			

CI, confidence interval; CIS, cerebrovascular ischemic stroke; OR, odds ratio. *P*<0.05 is considered statistically significant.

Results

Participant characteristics

We studied 100 patients with ischemic stroke and 100 controls, without any statistically significant difference between both groups regarding age or sex. Ischemic stroke group showed significant higher serum PCSK9 level, serum total cholesterol, LDL-C, TG, and VLDL levels ($P < 0.001$each) compared with controls (Table 1).

Genetic analysis of the E670GSNP of *PCSK9* gene

Genetic analysis of the E670G SNP of *PCSK9* gene showed that the distribution of different genotyping (Fig.1) in patients with ischemic stroke was following the Hardy-Weinberg equation. The PCR amplification followed by 2% gel electrophoresis demonstrated 169 bp fragment. Digestion of 169 bp fragment by *MnI1* restriction enzyme followed by 10% polyacrylamide gel resulted in two fragments (100 and 69 bp) in the case of AA genotype. The GG genotype was identified by the absence of the restriction site and one fragment 169 bp.

The frequency of genotypes and the alleles of *E670G SNP* of *PCSK9* gene are showed in Table 2. The AA genotype is the normal reference variance for this SNP, and it was more distributed in controls (68%). Both of the heterozygous variant AG and the minor G allele showed higher distribution in ischemic stroke group. There was a significant difference between patients with ischemic stroke and healthy controls. The heterozygous (AG) carriers were at risk of developing ischemic stroke (OR = 2.234, 95%CI = 1.2323–4.0509, $P = 0.0081$) compared with healthy controls. The G allele of *E670G* SNP of *PCSK9* gene was associated with increasing ischemic stroke risk, as the frequency of minor (G) allele distribution was significantly higher in patients with cerebrovascular ischemic stroke (OR = 1.6849, 95%CI = 1.0557–2.7150, $P = 0.0321$).

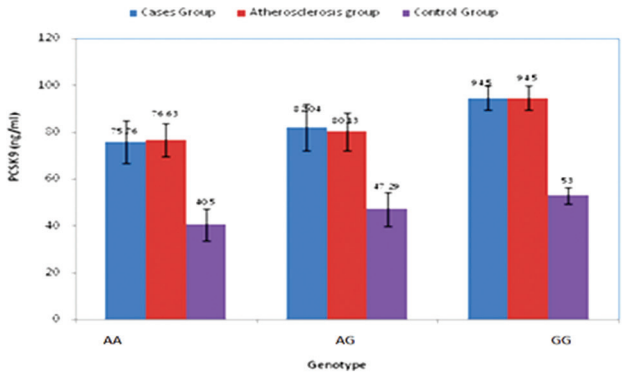
The AG genotype showed an association with the risk of atherosclerotic ischemic stroke (Table 3) (OR = 2.428, 95% CI = 1.256–4.692, $P = 0.0083$) compared with controls. The minor (G) allele was associated with increased risk of atherosclerotic ischemic stroke (OR = 1.8981, 95% CI = 1.133–3.3179, $P = 0.0149$). Moreover, both minor allele (G) and dominant model of inheritance (AA vs. AG + GG) showed an association with the risk of atherosclerotic cerebrovascular

Table 3 Genotyping and allelic frequencies distribution of E670G SNP of *PCSK9* gene in control and ischemic stroke subgroups

Genotype	Control group (*n*=100)	CIS cases (*n*=100)							
		Atherosclerotic ischemic stroke subgroup				Nonatherosclerotic ischemic stroke subgroup			
		n=68	OR	95% CI	*P*	*n*=32	OR	95% CI	*P*
AA*	68 (68.0)	32 (47.1)	Ref	Ref	Ref	18 (56.3)	Ref	Ref	Ref
AG	28 (28.0)	32 (47.1)	2.4286	1.2569-4.6924	0.0083	14 (43.8)	1.8889	0.8273-4.3125	0.131
GG	4 (4.0)	4 (5.9)	2.1250	0.4993-9.0430	0.3077	0	0.4114	0.0212-7.9919	0.557
Recessive AA + AG	96 (96)	64 (94.11)	1.5	0.3620-6.215	0.576	32 (100)	0.3299	0.0173-6.295	0.461
GG	4 (4)	4 (5.88)				0			
Dominant AA	68 (68)	32 (47)	2.3906	0.126-4.511	0.0071	18 (56.3)	1.652	0.7315-3.734	0.227
AG + GG	32 (32)	36 (52.94)				14 (43.8)			
Alleles A*	164 (82)	96 (70.58)	1.8981	1.1331-3.179	0.0149	50 (78.12)	1.2756	0.6374-2.5528	0.491
G	36 (18)	40 (29.41)				14 (21.87)			

Data is represented as number and percentage. CI, confidence interval; CIS, cerebrovascular ischemic stroke; OR, odds ratio. *P*<0.05 is considered statistically significant.

Figure 2



Clustered column chart showing serum PCSK9 (ng/ml) in cases ischemic stroke (*n*=100), atherosclerotic ischemic stroke (*n*=68), and controls (*n*=100) in relation to different genotyping of E670G SNP. SNP, single nucleotide polymorphism.

ischemic stroke (OR = 1.898, 95%CI = 1.133–3.179, *P* = 0.014) (OR = 2.39, 95%CI = 0.1266–4.511, *P* = 0.0071, respectively) in comparison with controls.

Association of serum PCSK9 and other laboratory ischemic stroke indices with different genotyping variants of E670G SNP of *PCSK9* gene

Significant associations were found among different genotyping carriers of E670G SNP of *PCSK9* gene and circulating plasma PCSK9, TC, and LDL-C levels in ischemic stroke group (*n* = 100) and atherosclerotic ischemic stroke subgroup (*n* = 68 with *P* < 0.001 each) (Table 4). The highest circulating PSCK9 levels were observed in GG carriers (Fig.2) either in ischemic stroke (*n* = 100) or atherosclerotic ischemic stroke subgroups (*n* = 68, *P* < 0.001 each), whereas carriers of AA genotype had significant lower levels of serum PCSK9, TC, LDL-C, TG, and VLDL in patients with ischemic stroke and patients with atherosclerotic ischemic stroke (Table 4).

Correlation studies between serum PSCK9 levels and other laboratory markers

Pearson's correlation studies for our results reveled a significant positive correlation between serum PSCK9 levels and each of total cholesterol (r = 0.763, P > 0.001), (r = 0.694, P < 0.001) and LDL-C (r = 0.742, P > 0.001), (r = 0.727, P > 0.001) in both ischemic stroke group and the atherosclerotic ischemic stroke subgroup, respectively.

Serum levels of TG were significantly correlated with serum PSCK9 in ischemic stroke group only (r = 0.298, P = 0.003) but not in ischemic atherosclerotic subgroup (r = 0.100, P = 0.419), and also, VLDL levels (r = 0.233, P = 0.020) were significantly correlated with serum PSCK9 in ischemic stroke group. Serum high-density lipoprotein-cholesterol levels were not correlated with serum PSCK9 levels in both ischemic stroke group (r=–0.140, P = 0.165) and ischemic atherosclerotic subgroup (r=–0.212, P–=0.082) (Table 5).

Discussion

In the present study, we attempted to evaluate the interplay between circulating PCSK9 levels and *E670G* polymorphism of *PCSK9* gene with the risk of ischemic stroke and atherosclerotic ischemic stroke in Egyptian patients. The frequency of minor G allele in our control group was 18%, which is different from previous studies that reported a lower frequency of G allele, i.e., 3.6% in Malian[22] and 7.3% in Tunisian [13]. This could be attributed to the racial, geographic, and environmental variations. The results of the present study revealed that the minor (G) allele of *E670G* polymorphism of *PCSK9* gene was significantly higher in ischemic stroke than controls. The high distribution of *G* allele of *E670G* polymorphism of *PCSK9* gene in ischemic stroke group in our study was mentioned in different studies among different populations [23–27];

Table 4 Relation between E670G SNP of *PSCK9* gene different genotypes and biochemical data in different groups

	Genotypes in ischemic stroke cases (*n*=100)			ANOVA	
	AA (*n*=50)	AG (*n*=46)	GG (*n*=4)	*F*	*P*
PCSK9 (ng/ml)	75.76±8.98	82.04±10.07[a]	94.50±5.20[a,b]	10.692	<0.001**
TC (mg/dl)	305.06±72.74	336.95±77.76[a]	523.36±7.51[a,b]	16.664	<0.001**
HDL-C (mg/dl)	48.52±13.95	43.98±20.37	53.67±12.70	1.180	0.312
LDL-C (mg/dl)	215.28±75.86	240.67±73.42	410.21±29.80[a,b]	13.220	<0.001**
TG (mg/dl)	217.65±63.37	270.70±88.09[a]	240.92±17.34	5.981	0.004*
VLDL (mg/dl)	41.27±14.37	52.30±17.27[a]	59.50±9.59[a]	7.244	<0.001**
	Genotypes in atherosclerotic ischemic stroke (*n*=68)			F	P
	AA (*n*=32)	AG (*n*=32)	GG (*n*=4)		
PCSK9 (ng/ml)	76.63±7.00	80.13±8.09	94.50±5.20[a,b]	10.525	<0.001**
TC (mg/dl)	308.88±69.85	326.67±73.57	523.36±7.51[a,b]	16.716	<0.001**
HDL-C (mg/dl)	50.85±15.10	49.48±19.20	53.67±12.70	0.131	0.878
LDL-C (mg/dl)	213.23±72.66	226.51±69.38	410.21±29.80[a,b]	14.338	<0.001**
TG (mg/dl)	219.58±59.42	251.60±88.66	240.92±17.34	1.515	0.227
VLDL (mg/dl)	44.81±14.24	50.69±16.85	59.50±9.59	2.272	0.111

Data represented as mean±SD. ANOVA test used. ANOVA, analysis of variance; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglyceride; VLDL, very low-density lipoprotein cholesterol. aSignificant difference vs AA at *P*<0.05. bSignificant difference significant vs AG at *P*<0.05. *P*<0.05 is considered statistically significant. **P*<0.05. ***P*<0.001.

Table 5 Correlation between PCSK9 and laboratory data in ischemic stroke cases (*n*=100)

Ischemic stroke group	PCSK9 (ng/ml)	
	r	*P*
TC (mg/dl)	0.736	<0.001**
HDL-C (mg/dl)	–0.140	0.165
LDL-C (mg/dl)	0.742	<0.001**
TG (mg/dl)	0.298	0.003*
VLDL (mg/dl)	0.233	0.020*

Person's correlation coefficient (*r*). HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglyceride; VLDL, very-low-density lipoprotein cholesterol. *P*<0.05 is considered statistically significant.

however, these studies failed to prove a significant association between E670G polymorphism of *PCSK9* gene and ischemic stroke. Our findings of a significant association are consistent with a recent meta-analysis, which evaluated seven case–control studies encompassing 1897 cases and 2119 controls, and showed that *E670G* polymorphism of *PCSK9* gene might contribute to the susceptibility of ischemic stroke in different populations like Chinese, Japanese, Tunisian, and Belgium people [28].

Our results showed also that carriers of the G allele of *E670G* polymorphism of *PCSK9* gene had significantly higher serum LDL-C and total cholesterol levels in atherosclerotic ischemic stroke, suggesting that this gain-of-function mutation plays an important direct role in deregulation of lipid metabolism and in the pathogenesis of atherosclerosis. Kotlęga *et al.*[29] stated that the G variant of E670G polymorphism of *PCSK9* gene could be an independent determinant of plasma LDL-C levels and hence the severity of atherosclerosis. Evans and Beil[21] also concluded that the E670G SNP in the *PCSK9* gene is associated with increased LDL-C in men. Notably, Qui *et al.*[6] also revealed that *E670G* polymorphism of *PCSK9* gene was closely related to higher LDL-C and TG levels, although they could not decipher whether this relationship is causal or concomitant with increased LDL-C levels and cardiovascular risk. In contrast to our results, the study by Hsu *et al.*[30] reported that the G allele was associated with lower LDL-C. Moreover, Aung *et al.*[15] reported that the *E670G* polymorphism of *PCSK9* gene did not associate with serum lipid levels. These discrepancies among studies may be owing to the sample size or different characteristics of the study population, such as age, sex, ethnicity, and/or environmental factors.

The present study showed higher PCSK9 serum level in atherosclerotic group compared with control. Our results reinforce the results of the study by Shapiro and Fazio [31], which ensured PCSK9 expression in endothelial cells, vascular smooth muscle cells, and in other areas of the human atherosclerotic plaque. Sun *et al.*[32] also confirmed our findings. They explained PCSK9 effect through a post-transcriptional pathway. They noted that PCSK9 regulates the level of LDLR and plays a major role in cholesterol homeostasis. In addition to the degradation of LDLR; PCSK9 may also modulate apoB100 degradation via autophagy flux in hepatocytes which may be another factor in atherosclerosis.

The atherosclerotic group had nonsignificant PCSK9 serum level when compared with nonatherosclerotic group. This can be explained by different functions of PCSK9, as it can work in different ways in case of ischemia through affecting platelets aggregation[33] and neuronal apoptosis [34].

Our finding of increased PCSK9 circulating levels in patients with ischemic stroke is supported by the findings of Ason *et al.*[35] and Tavori *et al.* [36], who reported that *PCSK9* increases hepatic apoB-containing lipoproteins in an LDLR-independent fashion. Levy *et al.*[37] had demonstrated that *PCSK9* regulates apoB48 secretion and cholesterol metabolism independently of the LDLR. Karagiannis *et al.*[38] observed that the LDL-C reduction induced by *PCSK9* inhibition likely comprises a main underlying mechanism for athero-protection. Rashid *et al.*[39] also found *PCSK9* stimulates intestinal microsomal triglyceride transfer protein independently of the LDLR. These effects confirmed by the attenuation of cardiovascular risk appeared with PCSK9 inhibition which beyond LDL-C lowering, these could hypothesize a possible anti-atherosclerotic benefit [25].

The findings of our study show that serum PSCK9 levels correlate with each of the TC, LDL-C, TG, and VLDL levels in patients with ischemic stroke. PCSK9 serum levels together with *PCSK9 E670G* gene variant are strong associating factors in the etiology of atherosclerosis, lipid levels, and the susceptibility to ischemic stroke and atherosclerotic ischemic stroke in Egyptian patients.

Conclusion

Serum PCSK9 levels were significantly higher in patients with ischemic stroke and those with atherosclerotic ischemic stroke and correlated with LDL-C level. Our results reinforce that PCSK9 protein is directly related to the pathogenesis of ischemic stroke and atherosclerotic ischemic stroke through LDL-C metabolism, thus mediating the initiation of atherosclerotic plaque. The G allele of E670G SNP *of PCSK9 gene* could be a genetic risk factor of ischemic stroke. Furthermore, carriers of the G allele of *E670G* SNP had significantly higher serum TC and LDL-C levels in atherosclerotic ischemic stroke subgroup, suggesting that this gain-of-function mutation plays an important direct role in the pathogenesis of lipid metabolism and on atherogenesis.

Limitations

Limitations of this study included limited data about the patients and control subjects, the retrospective design, and the between study heterogeneity. Another limitation of the study is that there were no sex-matched normal weight controls for each racial/ethnic group. The relationships between this SNP and lipid levels did not consider the confounding factors, such as age, sex, smoking, drinking, and other lifestyle factors in ischemic stroke group. Moreover, it is important to highlight the limitations of a small sample size.

Recommendations

Prospective studies are needed about the benefit of reduction of LDL-C and PCSK9 levels among carriers of AG genotype of E670G SNP of *PCSK9* gene. Moreover, further studies with larger sample size are required to confirm our results.

Finally, studies about the nonmetabolic effect of PCSK9 in cerebral ischemic stroke and peripheral ischemic diseases are needed to understand the other mechanisms of PCSK9 actions.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

References

1. Tian D, Yang Q, Dong Q, Li N, Yan B, Fan D. Trends in stroke subtypes and vascular risk factors in a stroke center in China over 10 years. Sci Rep 2018; 8:5037.
2. Khedr EM, Elfetoh NA, Al Attar G, Ahmed MA, Ali AM, Hamdy A, *et al.* Epidemiological study and risk factors of stroke in Assiut Governorate, Egypt: community-based study. Neuroepidemiology 2013; 40:288-294.
3. Soliman RH, Oraby MI, Fathy M, Essam AM. Risk factors of acute ischemic stroke in patients presented to Beni-Suef University Hospital: prevalence and relation to stroke severity at presentation. Egypt J Neurol Psychiatr Neurosurg. 2018; 54:8.
4. Wu N-Q, Li J-J. PCSK9 gene mutations and low-density lipoprotein cholesterol. Clinica Chimica Acta 2014; 431:148-153.
5. Yue Y-h, Liu L-y, Hu L, Li Y-m, Mao J-p, Yang X-y, *et al.* The association of lipid metabolism relative gene polymorphisms and ischemic stroke in Han and Uighur population of Xinjiang. Lipids Health Dis 2017; 16:120.
6. Qiu C, Zeng P, Li X, Zhang Z, Pan B, Peng ZY, *et al.* What is the impact of PCSK9 rs505151 and rs11591147 polymorphisms on serum lipids level and cardiovascular risk: a meta-analysis. Lipids Health Dis 2017; 16:111.
7. Farnier M. PCSK9: From discovery to therapeutic applications. Arch Cardiovasc Dis 2014; 107:58-66.
8 O'Connell E M, Lohoff FW. Proprotein convertase subtilisin/Kexin Type 9 (PCSK9) in the brain and relevance for neuropsychiatric disorders. Frontiers in neuroscience 2020; 14:609.
9. Li J-J, Li S, Zhang Y, Xu R-X, Guo Y-L, Zhu C-G, *et al.* Proprotein convertase subtilisin/kexin type 9, C-reactive protein, coronary severity, and outcomes in patients with stable coronary artery disease: a prospective observational cohort study. Medicine 2015; 94:52.
10. Frostegard J, Liu A. 3856PCSK9 plays a novel immunological role in oxidized LDL-induced dendritic cell maturation and T cell activation from human blood and atherosclerotic plaque. Eur Heart J 2017; 38(Suppl 1):ehx504.3856.
11. Liu A, Frostegard J. Oxidized LDL induced dendritic cell maturation and T cell activation – a novel immunological role of PCSK9. Atherosclerosis 2018; 275:E44.
12. Tang ZH, Li TH, Peng J, Zheng J, Li TT, Liu LS, *et al.* PCSK9: a novel inflammation modulator in atherosclerosis? J Cell Physiol 2019; 234:2345-2355.
13. Slimani A, Harira Y, Trabelsi I, Jomaa W, Maatouk F, Hamda KB, *et al.* Effect of E670G polymorphism in PCSK9 gene on the risk and severity

of coronary heart disease and ischemic stroke in a Tunisian cohort. J Mol Neurosci 2014; 53:150-157.

14. Chen SN, Ballantyne CM, Gotto AM, Tan Y, Willerson JT, Marian AJ. A common PCSK9haplotype, encompassing the E670G coding single nucleotide polymorphism, is a novel genetic marker for plasma low-density lipoprotein cholesterol levels and severity of coronary atherosclerosis. J Am Coll Cardiol 2005; 45:1611-1619.

15. Aung LHH, Yin R-X, Miao L, Hu X-J, Yan T-T, Cao X-L, *et al*. The proprotein convertase subtilisin/kexin type 9 gene E670G polymorphism and serum lipid levels in the Guangxi Bai Ku Yao and Han populations. Lipids Health Dis 2011; 10:5.

16. Polisecki E, Peter I, Robertson M, McMahon A, Ford I, Packard C, *et al*. PROSPER Study Group Genetic variation at the PCSK9 locus moderately lowers low-density lipoprotein cholesterol levels but does not significantly lower vascular disease risk in an elderly population. Atherosclerosis 2008; 200:95-101.

17. Cai G, Yu L, Huang Z, Li L, Fu X. Serum PCSK9 levels, but not PCSK9 polymorphisms, are associated with CAD risk and lipid profiles in southern Chinese Han population. Lipids Health Dis 2018; 17:213.

18. Reda A, Elserafy AS, Farag E, Mostafa T, Farag N, Elbahry A, *et al*. Egyptian Association of Vascular Biology and Atherosclerosis (EAVA) consensus on the usage of proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors. Egypt Heart J 2020; 72:1-6.

19. Grant EG, Benson CB, Moneta GL, Alexandrov AV, Baker JD, Bluth EI, *et al*. Carotid artery stenosis: gray-scale and Doppler US diagnosis – Society of Radiologists in Ultrasound Consensus Conference. Radiology 2003; 229:340-346.

20. Warnick GR, Knopp RH, Fitzpatrick V, Branson L. Estimating low-density lipoprotein cholesterol by the Friedewald equation is adequate for classifying patients on the basis of nationally recommended cutpoints. Clin Chem 1990; 36:15-19.

21. Evans D, Beil FU. The E670G SNP in the PCSK9 gene is associated with polygenic hypercholesterolemia in men but not in women. BMC Med Genet 2006; 7:66.

22. Arama C, Diarra I, Kouriba B, Sirois F, Fedoryak O, Thera MA, *et al*. Malaria severity: possible influence of the E670G PCSK9 polymorphism: a preliminary case-control study in Malian children. PLoS One 2018; 13:e0192850.

23. Huang C-C, Fornage M, Lloyd-Jones DM, Wei GS, Boerwinkle E, Liu K. Longitudinal association of PCSK9 sequence variations with low-density lipoprotein cholesterol levels: the Coronary Artery Risk Development in Young Adults Study. Circulation 2009; 2:354-361.

24. Hopewell JC, Malik R, Valdés-Márquez E, Worrall BB, Collins R, ISGC MCot. Differential effects of PCSK9 variants on risk of coronary disease and ischaemic stroke. Eur Heart J 2017; 39:354-359.

25. Han D, Ma J, Zhang X, Cai J, Li J, Tuerxun T, *et al*. Correlation of PCSK9 gene polymorphism with cerebral ischemic stroke in Xinjiang Han and Uygur populations. Med Sci Monit. 2014; 20:1758.

26. Han D-F, Ma J-H, Hao C-G. Association and differences in genetic polymorphisms in PCSK9 gene in subjects with lacunar infarction in the Han and Uygur populations of Xinjiang Uygur Autonomous Region of China. Neural Regen Res 2017; 12:1315.

27. Barboza MAA, Serrano F, Jara A, Silva S, Arauz A. Abstract TP206: association of a polymorphism from the protein convertase subtilisin kexin Type 9 in atherosclerotic ischemic stroke in two Latin American populations. Stroke 2018; 49(Suppl_1):ATP206-ATP206.

28. Au A, Griffiths LR, Cheng K-K, Kooi CW, Irene L, Wei LK. The influence of OLR1 and PCSK9 gene polymorphisms on ischemic stroke: evidence from a meta-analysis. Sci Rep 2015; 5:18224.

29. Kotlęga D, Gołąb-Janowska M, Masztalewicz M, Ciećwież S, Nowacki P. Association between selected gene polymorphisms and statin metabolism, risk of ischemic stroke and cardiovascular disorders. Advances in Hygiene & Experimental Medicine/Postepy Higieny i Medycyny Doswiadczalnej 2016;70.

30. Hsu L-A, Teng M-S, Ko Y-L, Chang C-j, Wu S, Wang C-L, *et al*. The PCSK9 gene E670G polymorphism affects low-density lipoprotein cholesterol levels but is not a risk factor for coronary artery disease in ethnic Chinese in Taiwan. Clin Chem Lab Med 2009; 47:154-158.

31. Shapiro MD, Fazio S. PCSK9 and atherosclerosis-lipids and beyond. J Atheroscler Thromb 2017; 24:RV17003.

32. Sun H, Krauss RM, Chang JT, Teng B-B. PCSK9 deficiency reduces atherosclerosis, apolipoprotein B secretion, and endothelial dysfunction. J Lipid Res 2018; 59:207-223.

33. Navarese EP, Kolodziejczak M, Winter MP, Alimohammadi A, Lang IM, Buffon A, *et al*. Association of PCSK9 with platelet reactivity in patients with acute coronary syndrome treated with prasugrel or ticagrelor: the PCSK9-REACT study. Int J Cardiol 2017; 227:644-649.

34. Apaijai N, Moisescu DM, Palee S, McSweeney CM, Saiyasit N, Maneechote C, *et al*. Pretreatment with PCSK9 inhibitor protects the brain against cardiac ischemia/reperfusion injury through a reduction of neuronal inflammation and amyloid beta aggregation. J Am Heart Assoc. 2019; 8:e010838.

35. Ason B, van der Hoorn JW, Chan J, Lee E, Pieterman EJ, Nguyen KK, *et al*. PCSK9 inhibition fails to alter hepatic LDLR, circulating cholesterol, and atherosclerosis in the absence of ApoE. J Lipid Res 2014; 55:2370-2379.

36. Tavori H, Fan D, Blakemore JL, Yancey PG, Ding L, Linton MF, *et al*. Serum proprotein convertase subtilisin/kexin type 9 and cell surface low-density lipoprotein receptor: evidence for a reciprocal regulation. Circulation 2013;127:2403-2413.

37. Levy E, Ouadda ABD, Spahis S, Sane AT, Garofalo C, Grenier É, *et al*. PCSK9 plays a significant role in cholesterol homeostasis and lipid transport in intestinal epithelial cells. Atherosclerosis 2013; 227:297-306.

38. Karagiannis AD, Liu M, Toth PP, Zhao S, Agrawal DK, Libby P, *et al*. Pleiotropic anti-atherosclerotic effects of PCSK9 inhibitors from molecular biology to clinical translation. Curr Atheroscler Rep 2018; 20:20.

39. Rashid S, Tavori H, Brown PE, Linton MF, He J, Giunzioni I, *et al*. Proprotein convertase subtilisin kexin type 9 promotes intestinal overproduction of triglyceride-rich apolipoprotein B lipoproteins through both low-density lipoprotein receptor–dependent and independent mechanisms. Circulation 2014; 130:431-441.