

DOI: <https://dx.doi.org/10.21123/bsj.2023.7696>

Gene Expression and Methylation Levels of *PCSK9* Gene in Iraqi Patients with Coronary Artery Disease

Marwa M. Al-Attar^{1*} 

Salwa J. Al-Awadi² 

Shaimaa Y. Abdulfattah³ 

¹Department of Biology, College of Science, Mustansiriyah University, Baghdad, Iraq.

²Department of Molecular and Medical Genetics Technologies, College of Biotechnology, Al-Nahrain University, Baghdad, Iraq.

³Biotechnology Research Center, Al-Nahrain University, Baghdad, Iraq.

*Corresponding author: narjees52012@gmail.com

E-mail addresses: Fsalwaj@yahoo.com , shaimaay31@gmail.com

Received 8/9/2022, Revised 22/11/2022, Accepted 24/11/2022, Published Online First 20/4/2023,
Published 01/12/2023



This work is licensed under a [Creative Commons Attribution 4.0 International License](https://creativecommons.org/licenses/by/4.0/).

Abstract:

The expression of the Proprotein Convertase Subtilisin/Kexin Type 9 gene (*PCSK9*) is inextricably related to lipid levels and a risk of atherosclerotic coronary artery disease (ASCAD). The present study aims to measure the quantity of *PCSK9* gene expression and the effect of methylation on its expression level taking part in the pathogenesis of acute coronary artery disorder.

A current study included 150 subjects from the Iraqi population, 100 ASCAD patients and 50 healthy controls. The concentration of *PCSK9* in each serum sample was determined by the ELISA technique, the expression levels of the *PCSK9* gene in whole blood were estimated by RT-qPCR – Quantitative Reverse Transcription PCR method, and DNA methylation level detection in *PCSK9* gene by using High Resolution Melting Analysis (HRM) technique.

The expression level of the *PCSK9* gene was 6.06 ± 1.84 in ASCAD patients group compared with a control group 1.00 ± 0.19 . That indicates the up-regulation of the *PCSK9* gene in patient with atherosclerosis. In addition, the *PCSK9* concentration was higher in patient group in comparison to the healthy controls group $P = < 0.01$. However, *PCSK9* methylation levels, a highly significant distinction between the two study groups $p = 0.002$. In conclusion, serum *PCSK9* levels are associated with coronary artery lesions as shown in receiver operating characteristic (ROC) analysis. Suggests that, serum *PCSK9* might be a good indicator of coronary artery disease development.

Keywords: Atherosclerotic coronary artery disease, ELISA, Gene expression, Methylation, *PCSK9*, RT-qPCR.

Introduction:

Atherosclerosis (AS) coronary artery disease is a lipid-derived inflammatory disease that includes multicellular alteration it is characterized by plaque formation and it reasons millions of death globally¹. These plaques are primarily composed of lipids, which cause an inflammatory response, resulting in disorderly flow and ASCAD². Many risk factors for ASCAD, some of which can be controlled, while others cannot. Risk factors that can be controlled (modifiable) are: High blood pressure, high levels of cholesterol, smoking, diabetes, being obese, insufficient physical activity, unhealthy eating habits, and stress are all risk factors. Those who are uncontrollable (conventional) are: Gender

(men are generally at higher risk of CAD); age (simply getting older raises the risk); as well as family history³. The initial soft lesion is composed of extracellular fat deposits, foam cells, and a small number of platelets⁴. In Iraq, as stated by the world health organization (WHO), CVD is the major cause of mortality in Iraq exceeding 18.50% of total mortality in 2017 and Iraq ranked the 19th internationally⁵. According to a previous study documented by⁶, who reported that an unexpectedly high prevalence of coronary atherosclerosis in Iraqi men is higher than in women, which is the highest in Asia when compared to results of other similar studies conducted in other countries. The *PCSK9* is a serine

protease that has recently received a lot of attention due to its important role in the regulation of plasma low density lipoprotein-cholesterol (LDL-C) levels and in determining (ASCAD) risk^{7, 8}. PCSK9 promotes low density lipoprotein-receptor (LDL-R) degradation in the liver via an unknown posttranscriptional mechanism. Some sequence variations of the *PCSK9* gene were found in the large long-term atherosclerosis risk in communities study, this is related to low LDL-C levels and a lower risk of coronary events. Other sequence variants, on the other hand, have been linked to the development of premature atherosclerosis, since mutations in *PCSK9* can cause severe hypercholesterolemia. In addition, the measurement of PCSK9 serum levels is a further important biomarker for atherosclerosis to detect the disease course and respond to therapy⁹. DNA methylation is an epigenetic alteration in human DNA that involves a chemical modification (substitution mutation) by the additional methyl group CH₃ to 5' carbon of the pyrimidine ring (5'-position of cytosine residues) and because of the changes caused by this process, the necessary proteins are unable to bind to the DNA molecule for initiate transcription and translation¹⁰. Compatible with a recent study, we discovered that methylated *PCSK9* gene at promoter region in CAD patients has 3.72-fold lower expression and 85 percent lower serum protein levels of PCSK9 than CAD patients with unmethylated DNA¹¹. This study aimed to evaluate *PCSK9* gene methylation, gene expression and quantification of serum levels of PCSK9 regulatory protein (affect lipid levels) in patients with ASCAD.

Materials and Procedures:

Participant in the study:

This study included one hundred fifty participants, ages between 30 to 65 years both male and female. Hundreds of patients had the clinical appearance of ASCAD, as well as undergoing a medical and physical examination and documented through coronary angiography, and fifty healthy controls were chosen at random, clinical examination revealed no signs of cardiovascular disease. Subjects were enlisted to help with diagnosis and treatment from the Iraqi Center of Heart Disease in Baghdad, Iraq. The study was ethically approved by the Ministry of Health in Iraq (decree order 3310 on 08/12/2021), through the Scientific Committee of the College of Biotechnology, Al-Nahrain University, carried out between December 2021 and May 2022. Patients diagnosed with ASCAD with TC usually greater than 200mg/dl and LDL-C greater than 160mg/dl (hypercholesterolemia),

triglycerides (TG) less than 200 mg/dl whose other lipid levels were normal according to the American heart association (AHA) guidelines, coronary angiography technique should be included with criteria equal to or more than 50 percent stenosis of at least one major coronary vessel due to atherosclerosis and confirmed by two experienced cardiologists ASCAD, and normolipidemic subjects with LDL-C less than 130 mg/dl and TG less than 150 mg/dl⁴. Exclusion criteria were: subjects with the presence of hypertension were previously diagnosed to have hypertension by a clinician. Diabetes mellitus was defined as the use of diabetes medications or a fasting blood glucose level equal to or more than 126 mg/dl. To avoid inaccuracy in the results, hypertriglyceridemia, renal, liver, or thyroid disease, autoimmune disease, malignant tumor, congenital heart disease (CHD), and Patients who lacked basic information were barred from participation.

Collection of blood samples:

The blood samples were separated into two groups; First group put into the serum separating tube was used to measure the concentration of PCSK9 protein by ELISA test. The second group of blood was placed in EDTA-containing tubes and separated into two parts for genomic DNA extraction by using *EasyPure*® Blood Genomic DNA Kit (Catalog No.EE121) and RNA extraction by using the *TransZol* Up Plus RNA Kit, then preserved at -20 °C.

Biochemical analysis:

Enzyme Linked Immunosorbent Assay (ELISA) test is a method of quantitative sandwich enzyme immunoassay, the microplate has been pre-coated with a monoclonal antibody (mAb) specific for human PCSK9¹². The PCSK9 concentration in each serum sample was represented as ng/ml for the comparison with the results with those of controls concentration and the detection ranges: 177ng/ml-460ng/ml, ELISA kits used in this study produced by (R&D/ USA).

Quantitative Reverse Transcriptase PCR (RT-qPCR) for Relative PCSK9 Gene Expression:

Quantitative reverse transcription PCR (RT-qPCR) involves two steps, the first convert in total RNA to cDNA was performed by using *EasyScript*® One-Step gDNA Removal and cDNA Synthesis SuperMix Kit through utilizing PCR (thermocycler) machine. The PCR Condition for cDNA synthesis is programmed as, 25 °C for 10 min for random primer binding, 42°C for 15 min for reverse transcriptase enzyme activation and Oligo(dT) primer binding and 85 °C for 5 sec for

enzyme inactivation. The second step RT-qPCR analysis was used to evaluate the gene expression levels of target gene by utilizing Qiagen Rotor gene Q Real-time PCR System. The components for RT-qPCR analysis were the cDNA was mixed with the SYBER Green master mix provided by *TransStart*® Top Green qPCR SuperMix Kit, in addition specific forward and reverse primers (primers and housekeeping genes utilized in present study with their sequences are summarized in Table 1) and complete the volume of reaction to 20 µl by using nuclease free water and the RT-qPCR program of *PCSK9*, and *GAPDH* genes as the following condition: the Enzyme activation step 94 °C for 90 sec for 1cycle in hold 1, followed denaturation step 94 °C for 5 sec, then annealing 58 °C for *PCSK9*

and 64 °C for *GAPDH* genes for 15 sec and extension 72 °C for 20 sec for 40 cycles in hold 2, followed dissociation 55-95 °C for 1 min for 1 cycle. Each reaction was performed in duplicate and included a non-template control (NTC), non-amplification control(NAC) and non-primer control (NPC) as negative controls were done in separate run to ensure efficacy of (primers , template and master mix). The relative cycle threshold ($2^{-\Delta\Delta Ct}$) method was used to calculate fold changes in the quantified expression of mature RNAs¹³. The target gene expressions were normalized by using housekeeping gene *Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)* as a reference gene to obtain accurate Ct values from the Quantitative real time PCR (qPCR) instrument¹⁴.

Table 1. Primers of *PCSK9* gene expression used in the study.

Primer	Sequence (5'→3')	Product size bp	Reference
<i>PCSK9</i> Expression	F:5'- ACCTCACCAAGATCCTGCAT -3' R:5'-TAGTCGACATGGGGCAACTT -3'	106	Designed for the current study
<i>GAPDH</i>	F:5 ' -GAAATCCCATCACCATCTTCCAGG -3 ' R:5 '-GAGCCCCAGCCTTCTCCATG -3 '	120	(Zeng <i>et al.</i> , 2017)

DNA Methylation of *PCSK9* Gene:

The extracted genomic DNA was utilized for DNA Methylation of *PCSK9* by using the High-Resolution Melting (HRM) technique. The HRM-PCR template was prepared by Bisulfite conversion assay, utilizing the EpiTect-Fast DNA Bisulfite Kit. Sodium bisulfite was used to modify genomic DNA, converting all unmethylated cytosine to uracil, while methylated cytosine remain unchanged. After completion of the bisulfite conversion, the converted DNA is ready to be used in the HRM-PCR technique for determining the

percentage of methylated and un-methylated cytosine in DNA¹². A primer sequence utilized in the present study as shown in Table 2, was designed for current study by using bioinformatics tools (Meth Primer and Beacon Designer software 8.21) (<http://epidesigner.com>) for methylation of the human *PCSK9* promoter region and a region was selected from 2768-2997 bp provided by Alpha-DNA Company (Canada). The choice of this region in the promoter of *PCSK9* is because this region within the transcription start site (TSS) is rich in CpG island=15.

Table 2. Primer sequence of DNA methylation of *PCSK9* gene used in the study.

Primer	Sequence (5'→3')	Product size bp	Reference
<i>PCSK9</i> Methylation	F:5'- GAGAGGAGGAGTTTTAGGG -3' R:5'-AAAACCTTCTAAAATATAAATACTTAA -3'	230	Designed for the current study

The HRM-PCR reaction mix for the *PCSK9* gene include: DNA template mix with Eva Green HRM-PCR Mix in addition to reverse and forward primers and completes the volume of reaction to 20 µl by using nuclease free water. The HRM-PCR program of *PCSK9* gene has the following condition: The denaturation step 95 °C for 12 min for 1cycle in hold 1, followed denaturation step 95 °C for 15 sec, then annealing 58 °C for 20 sec and extension 72 °C for 20 sec for 40 cycles in hold 2, followed dissociation 55-95 °C for 1 min for 1 cycle. The positive control (100 % methylated DNA) and negative control (100% unmethylated DNA) are

provided by EpiTect ® PCR control DNA kit that is used for normalization of reaction.

Statistical investigation:

IBM SPSS for Windows, version 26 was used to analyze the data (SPSS Inc. Chicago, Illinois, United States). The methods listed below were used to check the normality requirements of collected data: Skewness, Kurtosis, and Q-Q plot. In addition, the Kolmogorov-Smirnov and Shapiro-Wilk tests were used to determine normality. Frequencies and proportions were used to express categorical variables. The Chi-square test² was utilized to compare proportions. A mean and standard deviation of continuous variables were

calculated (SD). The student's t-test was applied to compare group differences. Variables with non-normal data distributions were log-transformed for analysis before being converted back to standard units. Mann-Whitney. The U nonparametric test was exercised to compare the two groups. Moreover, the Pearson Correlation produces a correlation coefficient (*r*) which measures the relationships between the studied parameters. The area under the curve (AUC) of some studied parameters is calculated using receiver operator characteristics (ROC). In addition, the best cut-off value (CV), sensitivity, and specificity were

studied. A *p*-value <0.05 was considered statistically significant^{15,16,17}.

Results and Discussion:

The study population's anthropometric distribution:

The result of anthropometric factors used in this study as shown in Table 3, was assessed according to a question addressed to the participants in this study. According to the age mean±SD of subjects, of a study group was 51.86 ± 9.76 for the patient group and 48.62 ± 10.09 for the control group, the result found that there was no significant association between both groups studied *p*= 0.06.

Table 3. The study groups' anthropometric characteristics.

Variables	Control (n=50)	Patient(n=100)	<i>P</i> value
Age (years)	48.62 ± 10.09	51.86 ± 9.76	0.06
Weight (Kg)	80.76 ± 13.70	81.41 ± 12.47	0.77
Height (Cm)	174.80 ± 9.29	170.37 ± 8.38	0.004
Ejection Fraction	63.28 ± 4.04	55.72 ± 10.29	0.00
Systolic pressure (mmHg)	11.94 ± 1.03	13.52 ± 2.14	0.00
Diastolic pressure (mmHg)	7.40 ± 1.04	8.27 ± 1.37	0.001
Gender			
Male	37 (74.00 %)	79 (79.00 %)	0.49
Female	13 (26.00%)	21 (21.00 %)	
Smoking Status			0.004
Smoker	8 (16.00%)	39 (39.00%)	
Non- smoker	42 (84.00%)	61 (61.00%)	
Alcohol status			0.152
Drinker	0 (0.00 %)	4 (4.00 %)	
Non-drinker	50 (100.00%)	96 (96.00%)	

The results displayed there was a statistically significant association between the two groups of study based on height 170.37 ± 8.38 vs. 174.80 ± 9.29, systolic pressure 13.52 ± 2.14 vs. 11.94 ± 1.03, diastolic pressure 8.27 ± 1.37 vs. 7.40 ± 1.04, ejection fraction 55.72 ± 10.29 vs. 63.28 ± 4.04 respectively *P*< 0.05, while no significant association *P*= 0.7 between two study groups based on weight 81.41 ± 12.47 vs. 80.76 ± 13.70. In addition, upon comparing the results of patient and control groups according to gender found the control group was made up of 74% men and 26% women, while the patient group was made up of 79% men and 21% women, there was no significant association between case and control groups *P*= 0.4. Furthermore, the smoking percentage in the patient group was 39% compared with the control group 16% and the non-smoker percentage in the case group was 61% compared with the control group 84%, there was a significant association between two groups *p*= 0.004, all males and females included either smokers or not smokers depending on patient data form. The percentage of the patient group depending on alcohol-consuming was 4% compared with the control group that was 0%, while

the patient group non-consumed 96 % more than the control group 100 %, but there were no significant differences between the two groups *P*=0.15.

The previous study displayed there was a significant association between smoking and the development of atherosclerosis, that risk was proportional to the number of cigarettes smoked per day¹⁸. Smoking contributed to the deaths of 2 million adults in Asia their age over 45 in 2004, which corresponded to a 1.4-fold increase in mortality¹⁹. Furthermore, 31% of smoking-related deaths were attributable to CVDs, the smoking-related CVD through many pathological processes including endothelial dysfunction, inflammation, altered lipid metabolism, and hypoxia because the chemicals in cigarette smoke cause the blood to thicken and form clots inside veins and arteries, blockage from a clot can lead to a heart attack and sudden death²⁰. Also, alcohol consumption (light to moderate) in the patient with CVD is associated with a lower incidence of cardiovascular and all-cause mortality²¹. Furthermore, study in 2009 found that 86.3% of the lower risk of CVD observed in moderate drinkers was explained by

alcohol's effects on lipids, glucose metabolism, inflammatory/hemostatic factors, and blood pressure. Nearly, 20% of the reduced risk of either total or CVD death among moderate drinkers was accounted for by these intermediate factors²²

The relationship between the mean of ASCAD patients and controls according to age there was no significant association, however, there was a significant relationship between the two study groups based on BMI means²³. In this study, the highest percentage of gender distribution was found in males than females, this is the same result of study prepared in 2015 that showed in their study the prevalence of ASCAD in Iraqi patients is more in men than women⁶. Men are more likely than women to develop atherosclerosis at a younger age because of female sex hormones' protective function, but this protection is lost after menopause such as progesterone, but other factors such as the way men deal with stress might also be of significance. However, as women get older their risk of developing atherosclerosis soon catches up with that of their male counterparts, especially after menopause²⁴. On the other hand, higher systolic (the pressure in the arteries when a heart beats) and diastolic blood pressure (the pressure in the arteries when a heart rests between beats) were associated

with a higher risk of CVD, but higher systolic blood pressure was associated with a higher risk of cardiovascular disease and little association of diastolic blood pressure with ASCAD, but not with CVD²⁵. According to ejection fraction (EF) Patients with EF $\leq 30\%$ but no other risk factor have low predicted mortality risk. Patients with EF $>30\%$ and other risk factors may have higher mortality and a higher risk of sudden death than some patients with EF $\leq 30\%$. Thus, risk of sudden death in patients with coronary disease depends on multiple variables in addition to EF²⁶. Ejection fraction is a measurement of the percentage of blood leaving a heart each time it contracts, it is usually measured only in the left ventricle. The left ventricle is the heart's main pumping chamber. It pumps oxygen-rich blood up into the body's main artery (aorta) to the rest of the body²⁷.

Biochemical analysis for estimation of Serum PCSK9 Level:

Evaluation of serum PCSK9 level between two study groups as shown in Fig.1. The results showed significant association between patient and healthy controls group according to PCSK9 serum levels $P < 0.01$, the mean for the case group 303.82 ± 49.94 and the controls group 181.15 ± 31.85 .

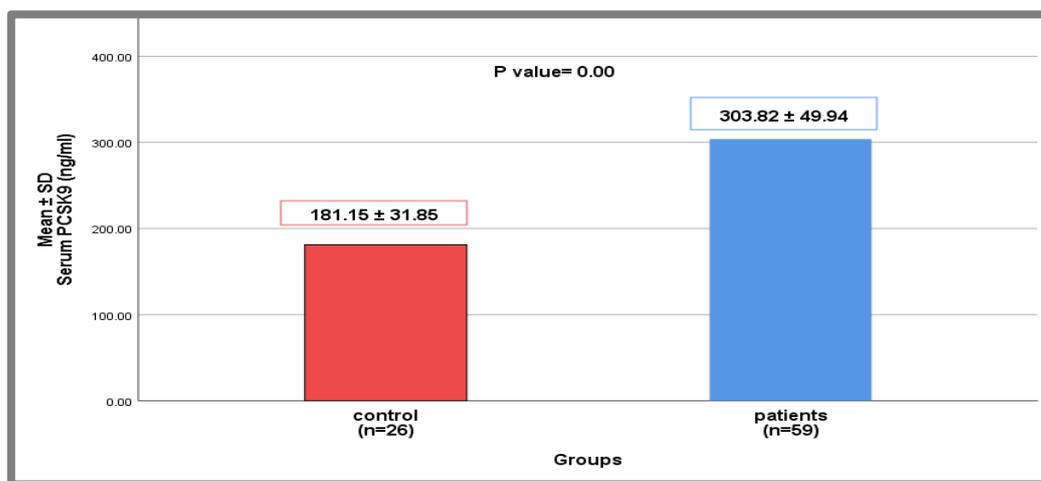


Figure 1. Serum PCSK9 level of the two study groups.

The increase in PCSK9 levels belongs to transcription factors SREBP-2 is a protein that binds the SRE in a PCSK9 proximal promoter, which then causes upregulation of the *PCSK9* gene and leads to increased circulating PCSK9 levels²⁸. In another similar study,^{29, 30} showed that statins medication inhibiting a rate-limiting enzyme in the synthesis of cholesterol, hydroxyl methylglutaryl-coenzyme A (HMG-CoA) reductase, reduces ER cholesterol, increases SREBP-2 processing, stimulates hepatic LDLR expression, and promotes

plasma LDL-C clearance. However, under these conditions, SREBP-2-induced PCSK9 expression likely reduces statin efficacy by stimulating PCSK9-mediated LDLR degradation.

Expression Level of *PCSK9* Gene:

As shown in Fig. 2 amplification curves for *PCSK9* and *GAPDH* genes by Quantitative real time PCR (qPCR) included all samples of study groups that were important for determining the Ct value of each sample, the image was taken directly from Rotor-Gene qPCR machine.

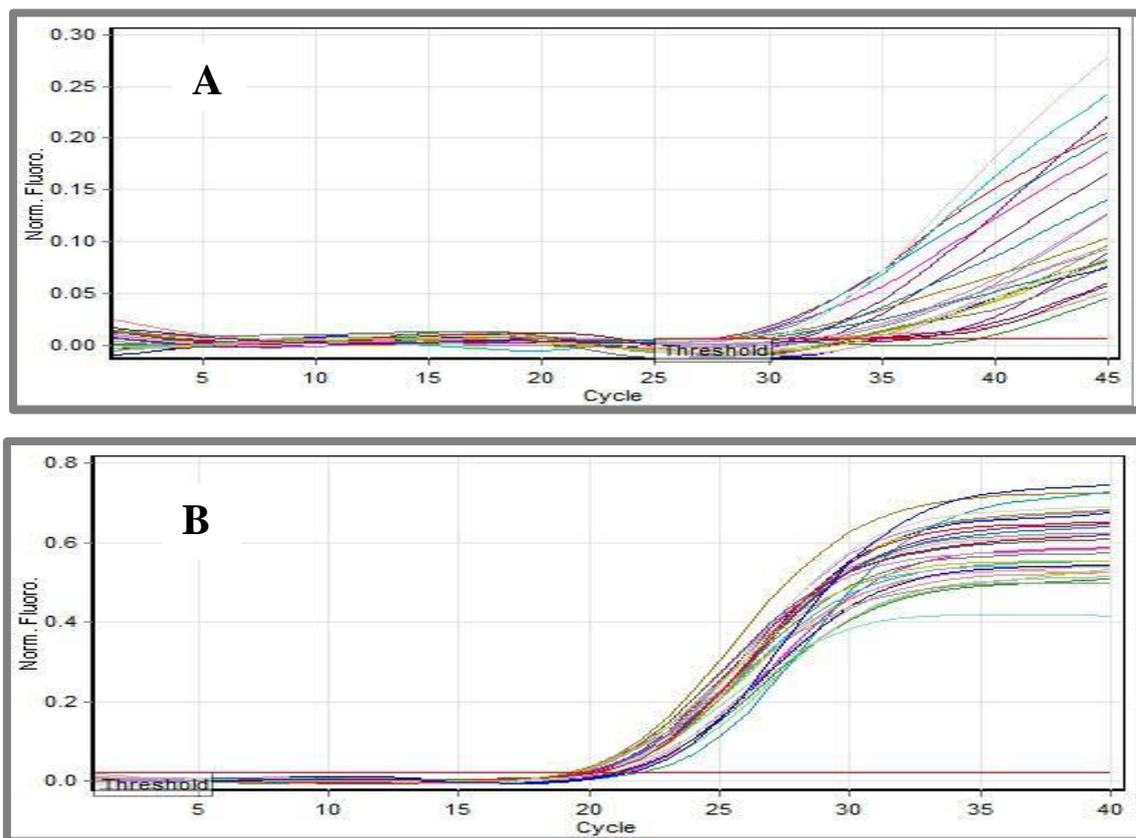


Figure 2. Amplification plots by qPCR included all study samples, A: for *PCSK9*; B: for *GAPDH*, that important for normalization to calculate ΔCt .

The gene expression for the *PCSK9* gene was analyzed by qPCR and used reference gene (*GAPDH*) for quantification and compare Ct-value

between two study groups in different stages of gene expression levels (Ct, ΔCt , $\Delta\Delta Ct$, and Fold Change) as shown in Table 4.

Table 4. *PCSK9* Gene Expression Fold Depending on $2^{-\Delta\Delta Ct}$ Method.

<i>PCSK9</i> Gene	Mean Ct of <i>PCSK9</i>	Mean Ct of <i>GAPDH</i>	ΔCt (Mean Ct of <i>PCSK9</i> - Mean Ct of <i>GAPDH</i>)	ΔCt Calibrator (Ct <i>PCSK9</i> - mean Ct <i>GAPDH</i>)	$\Delta\Delta Ct$	$2^{-\Delta\Delta Ct}$	Experimental group/ Control group	Fold gene expression \pm SE
Patient	33.19	20.45	12.74	18.6	-5.86	58.08	58.08/10.41	6.06 \pm 1.84
Control	35.18	19.96	15.22	18.6	-3.38	10.41	10.41/10.41	1.00 \pm 0.19

The findings of present study showed the Ct value of the *PCSK9* gene were 33.19 for patient group and 35.18 for control group and the Ct-value for *GAPDH* was 20.45 for patient group and 19.96 for control group. The ΔCt -value was calculated as result of the difference in Ct-value of *PCSK9* gene for patient and control and the Ct of *GAPDH* gene, these results found the expression level of *PCSK9* gene that was 6.06 ± 1.84 in the ASCAD patients' group depending on $2^{-\Delta\Delta Ct}$ and in the control group 1.00 ± 0.19 , that indicates the up-regulation of *PCSK9* gene in patients with atherosclerosis. The

concentration of intracellular cholesterol is the major regulator of the transcription of *PCSK9* via SREBP2 but insulin has also been shown to regulate the expression of *PCSK9* via SREBP-1c^{7, 31}.

DNA Methylation of *PCSK9* Using High Resolution Melting Analysis:

The results of the HRM-PCR analysis for *PCSK9* gene methylation levels for patients and healthy control groups presented in percentages graduating from 100% methylation to un-methylation that was used for normalization, as shown in Fig. 3A. While, Fig. 3B displayed the *PCSK9* gene comparative

curves by qPCR as a result of the change in fluorescence observed when double-stranded DNA with incorporated dye molecules dissociates into single-stranded DNA as the temperature of the

reaction is raised. In contrast, Fig. 3C, show amplification plots of *PCSK9* gene is useful for identifying value of methylation levels in the samples of ASCAD patient and control groups.

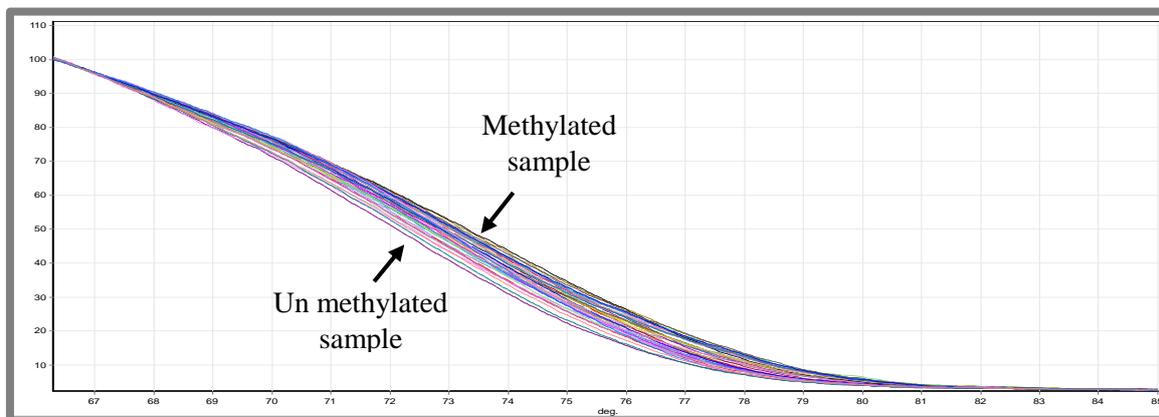


Figure 3A. Normalization graph for identification of a methylated and unmethylated pattern of *PCSK9* gene was done by HMR-PCR technique.

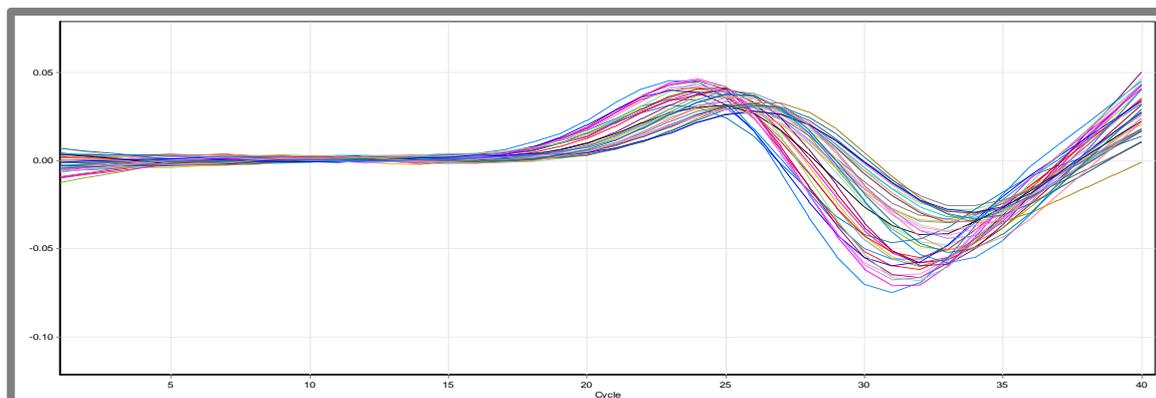


Figure 3B. *PCSK9* gene dissociation curves using qPCR. Melting temperature ranged from 80°C to 82°C.

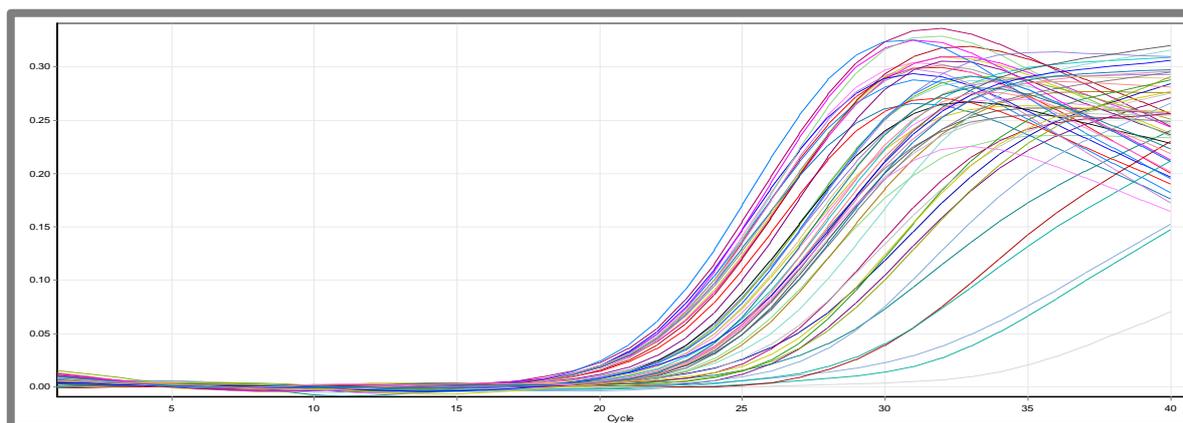


Figure 3C. Amplification plots of *PCSK9* gene using qPCR.

The results of DNA methylation patterns are displayed according to the diagnosis of a methylated pattern as shown in Fig. 4 For *PCSK9* methylation levels mean \pm SD, a highly significant relationship between two study groups $p=0.002$, the

mean for patients group 51.28 ± 21.32 that leading to indicate that hypomethylation has happened, while in the controls group hypermethylation was happened 62.72 ± 20.67 .

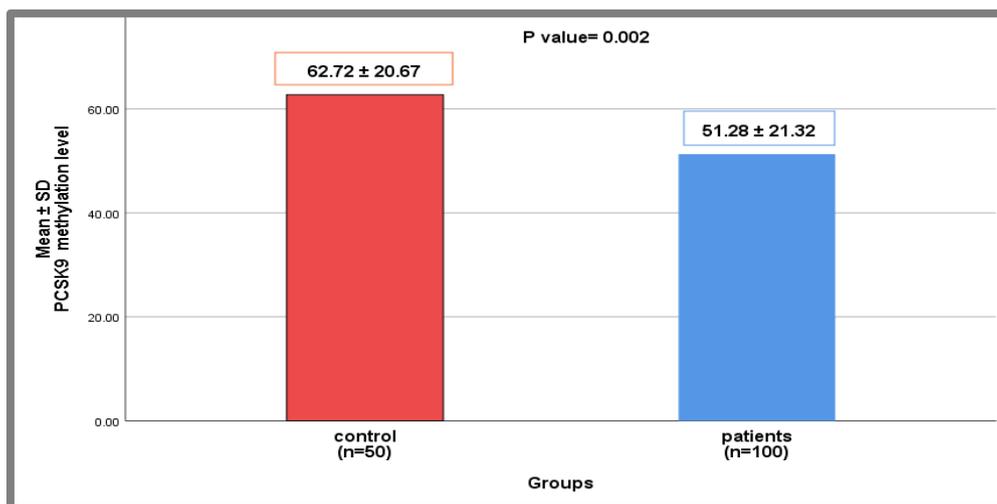


Figure 4. DNA methylation level of *PCSK9* gene of the studied groups.

DNA methylation regulates gene transcription where DNA hypermethylation usually leads to a long-term and stable gene suppression and DNA hypomethylation of the gene leads to gene expression^{12, 32}. The results of recent study found that were patients with methylated *PCSK9* promoter, the *PCSK9* gene expression and serum circulating levels are lower in those with unmethylated DNA, indicating a lower risk of disease¹¹.

Receiver Operating Characteristic (ROC) Analysis of study parameter:

A Receiver Operator Curve (ROC) was used to discover if the study parameter could be predictive of ASCAD⁴. As displayed in Table 5, the serum

PCSK9 level in a patient with atherosclerotic coronary artery disease has a high predictor of ASCAD with AUC=0.98, with the best Cut off value = 226.29ng/ml, sensitivity 93.20%, and specificity 100%, and displayed a significant difference P= 0.00. While the *PCSK9* methylation level in a patient with ASCAD has a good predictor of ASCAD with AUC=0.70, Cut off value = 55.95, sensitivity 64.20% and specificity 64.60%, with a significant difference P= 0.002. In addition, the *PCSK9* gene expression fold in a patient with ASCAD has a good predictor of ASCAD with AUC=0.79, Cut off value = 2.00, sensitivity 75.5% and specificity 76%, A result showed a significant difference P= 0.00.

Table 5. Receiver Operating Characteristic (ROC) analysis for study parameter in patients with ASCAD.

Parameters	AUC	P value	The best Cut off	Specificity%	Sensitivity %
<i>PCSK9</i> (ng/ml)	0.98	0.00	226.29ng/ml	100%	93.20%
$2^{-\Delta\Delta CT}$ <i>PCSK9</i>	0.79	0.00	2.00	76%	75.5%
<i>PCSK9</i> methylation	0.70	0.002	55.95	64.60%	64.20%

Conclusion:

In summary, the findings revealed that serum *PCSK9* levels are associated with the severity of coronary artery lesions, with an increase in ASCAD patients compared to control. The parameters employed in this study served as a sensitive marker for precise diagnosis of ASCAD and as a guide for future *PCSK9* inhibitor strategies.

Acknowledgements

The authors would like to thank the Iraqi Center of Heart Disease in Baghdad, Iraq for their facilities, which have helped enhance the quality of this work and all participants in this study.

Authors' declaration:

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript.
- The author has signed an ethics approval and consent for authors.
- Ethical Clearance: The project was approved by the local ethical committee in Ministry of Health, the Iraqi Center of Heart Disease (Ghazi Al - Hariri) Hospital/ Baghdad, Iraq (decree order 3310 on 08/12/2021).

Authors' contributions:

M M contributed to the methodology, investigation, data analysis and writing - original draft. S J and S Y contributed to design the study and supervising work methods, findings of this work, and writing.

References:

1. Tao J, Xia L, Cai Z, Liang L, Chen Y, Meng J, et al. Interaction Between microRNA and DNA Methylation in Atherosclerosis. *DNA Cell Biol.* 2021 Jan 19; 40(1): 1-15. <https://doi.org/10.1089/dna.2020.6138>.
2. Pahwa R, Jialal I. Atherosclerosis. In: StatPearls. Treasure Island (FL): StatPearls Publishing; 2022 Jan. <https://www.ncbi.nlm.nih.gov/books/NBK507799/>.
3. Abdulfattah SY, Al-Awadi SJ. ApoB gene polymorphism (rs676210) and its pharmacogenetics impact on atorvastatin response among Iraqi population with coronary artery disease. *J Genet Eng Biotechnol.* 2021 June 22; 19(1): 95. <https://doi.org/10.1186/s43141-021-00193-4>.
4. Abdulfattah SY, Al-Awadi SJ, Al-Saffar HB. Plasma oxidized low-density lipoprotein level and miRNA-146a gene expression, as a strong predictor for atherosclerotic coronary artery disease and its associated response to atorvastatin in a sample of the Iraqi population. *J Gene Rep.* 2020 Mar; 18: 2452-0144. <https://doi.org/10.1016/j.genrep.2019.100563>.
5. Abd RK, Abd SN, Raman V. Tracing the Risk Factors of Heart Diseases at Al-Nasiriyah Heart Center in Iraq. *J Cardiovasc Dis Res.* 2019; 10 (1): 31-34. <https://doi.org/10.5530/jcdr.2019.1.6>.
6. Al-Aamood AMK, Al-jubori NH, Al-Tmeme A. Prevalence of Coronary Atherosclerosis in Babylon Governorate; Histopathological, Postmortem Prospective Study. *Med J Bab.* 2015; 12 (2).
7. Shapiro MD, Fazio S. PCSK9 and atherosclerosis-lipids and beyond. *J Atheroscler Thromb.* 2017 Mar 9; 24(5): 462-472. <https://doi.org/10.5551/jat.RV17003>.
8. Zhang B, Lai X, Jia S. Epigenetics in Atherosclerosis a Clinical Perspective. *Discov Med.* 2015 Feb 19; 19(103), 73-80. <https://www.discoverymedicine.com/Bi-Kui-Zhang/2015/02/epigenetics-in-atherosclerosis-a-clinical-perspective/>.
9. Zhang PY. PCSK9 as a therapeutic target for cardiovascular disease. *Exp Ther Med.* 2017 Jan 17; 13(3), 810-814. <https://doi.org/10.3892/etm.2017.4055>.
10. Zayr FH, Dhahi MAR, Abdul-Rasheed OF, Hashim HM. DNA Methylation Patterns of Interferon Gamma Gene Promoter and Serum Level in Pulmonary Tuberculosis: Their Role in Prognosis. *Baghdad Sci J.* 2019 Mar. 17; 16(1(Suppl.)): 0178. <https://bsj.uobaghdad.edu.iq/index.php/BSJ/article/view/3230>
11. Shyamala N, Gundapaneni KK, Galimudi RK, Tupurani MA, Padala C, Puranam K, et al. PCSK9 genetic (rs11591147) and epigenetic (DNA methylation) modifications associated with PCSK9 expression and serum proteins in CAD patients. *J Gene Med.* 2021 Apr 22; 23(8): e3346. <https://doi.org/10.1002/jgm.3346>.
12. Al-Qazzaz HK, Al-Awadi SJ. Epigenetic alteration in DNA methylation pattern and gene expression level using H19 on oligospermia patients in Iraqi men. *J Gene Rep.* 2020 Sep; 20: 100780. <https://doi.org/10.1016/j.genrep.2020.100780>.
13. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-ΔΔCt} method. *Methods.* 2001 Dec; 25(4): 402-408. <https://doi.org/10.1006/meth.2001.1262>.
14. Albanna AMJ, Al-Layla AAH. Using Real-Time PCR to Investigate Some of Antibiotic Resistance Genes from *Streptococcus agalactiae* Isolates from ewe Mastitis cases in Nineveh province. *Baghdad Sci J.* 2020 Sep 8; 17(3(Suppl.)): 0931. <https://bsj.uobaghdad.edu.iq/index.php/BSJ/article/view/3123>
15. Al-Rahim AM, AlChalabi R, Al-Saffar AZ, Sulaiman GM, Albukhaty S, Belali T et al. Folate-methotrexate loaded bovine serum albumin nanoparticles preparation: an *in vitro* drug targeting cytokines overwhelming expressed immune cells from rheumatoid arthritis patients. *Anim Biotechnol.* 2021 Jul 28. <https://doi.org/10.1080/10495398.2021.1951282>.
16. Glover T, Mitchell K. An introduction to Biostatistics, 3ed Edition. Waveland press .Inc. 2008; (847): 634-0081; <https://waveland.com/Glover-Mitchell/Appendices.pdf>.
17. Forthofer RN, Lee ES. Introduction to biostatistics: a guide to design, analysis, and discovery, 2nd ed. Elsevier. 2014; p. 1-602. <https://doi.org/10.1016/C2009-0-03861-6>.
18. Zheng W, McLerran DF, Rolland BA, Fu Z, Boffetta P, He J. Burden of total and cause-specific mortality related to tobacco smoking among adults aged ≥ 45 years in Asia: a pooled analysis of 21 cohorts. *PLoS Med.* 2014 Apr 22; 11: e1001631. <https://doi.org/10.1371/journal.pmed.1001631>.
19. Kim M, Han CH, Lee MY. NADPH Oxidase and the Cardiovascular Toxicity Associated with Smoking. *Toxic Res.* 2014 Sep. 30(3): 149-157. <https://doi.org/10.5487/TR.2014.30.3.149>
20. Costanzo S, Castelnuovo AD, Donati MB, Iacoviello L, Gaetano GD. Alcohol Consumption and Mortality in Patients with Cardiovascular Disease. *J Am Coll Cardiol.* 2010 Mar 30; 55(13): 1339-1347. DOI: <https://doi.org/10.1016/j.jacc.2010.01.006>
21. Djoussé L, Lee IM, Buring JE, Gaziano JM. Alcohol Consumption and Risk of Cardiovascular Disease and Death in Women. *Circulation.* 2009 Jul 13. 120(3): 237-244. <https://doi.org/10.1161/CIRCULATIONAHA.108.832360>.
22. Hajar R. Risk factors for coronary artery disease: historical perspectives. *Heart views: the official j Gulf Hea Associa.* 2017 Sep; 18(3): 109-114. https://doi.org/10.4103/HEARTVIEWS.HEARTVIEWS_106_17.

23. Phulukdaree A, Khan S, Moodly D, Chuturgoon AA. GST polymorphisms and early – onset coronary artery disease in young. S Afr Med J. 2012; 102 (7): 15-19. <https://doi.org/10.7196/SAMJ.5520>
24. Whelton S, Deal JA, Zikusoka M, Jacobson LP, Sarkar S, Palella Jr, et al. Associations between lipids and subclinical coronary atherosclerosis. Aids. 2019 May 1; 33(6), 1053-1061. <https://doi.org/10.1097/QAD.0000000000002151>.
25. Chan I, Kwok K, Schooling M. The total and direct effects of systolic and diastolic blood pressure on cardiovascular disease and longevity using Mendelian randomization. Sci Rep. 2021 Nov 8; 11:21799. <https://doi.org/10.1038/s41598-021-00895-2>.
26. Alfred EB, Kerry L, Gail H, Luis P, John F, Michael RG, et al. Limitations of Ejection Fraction for Prediction of Sudden Death Risk in Patients With Coronary Artery Disease: Lessons From the MUSTT Study. J Am Coll Cardiol . 2007 Sep; 50(12): 1150–1157. <https://doi.org/10.1016/j.jacc.2007.04.095>.
27. Cikes M, Solomon SD. Beyond ejection fraction: an integrative approach for assessment of cardiac structure and function in heart failure. Eur Heart J. 2016 Jun 1; 37(21): 1642–1650. <https://doi.org/10.1093/eurheartj/ehv510>.
28. Dua P, Reeta KH. PCSK9 (Proprotein convertase subtilisin/ kexin type 9): A Narrative Review. Pract Cardiovas Sci. 2020 Dec 23; 6(3): 226-233. https://doi.org/10.4103/jpcs.jpcs_3_20.
29. Barale C, Melchionda E, Morotti A, Russo, I. PCSK9 biology and Its role in atherothrombosis. Int J Mol Sci. 2021 May 14; 22(11): 5880. <https://doi.org/10.3390/ijms22115880>.
30. Burke C, Dron S, Hegele A, Huff W. PCSK9 Regulation and Target for Drug Development for Dyslipidemia. Annu Rev Pharmacol Toxicol. 2017 Jul 31; 6(57): 223-244. <https://doi.org/10.1146/annurev-pharmtox-010716-104944>.
31. Pott J, Schlegel V, Teren A, Horn K, Kirsten H, Bluecher C, et al. Genetic regulation of PCSK9 (proprotein convertase subtilisin/kexin type 9) plasma levels and its impact on atherosclerotic vascular disease phenotypes. Circ Genom Precis Med. 2018 Jun 18; 11(5): e001992. <https://doi.org/10.1161/CIRCGEN.117.001992>.
32. Tabaei S, Tabaei S. DNA methylation abnormalities in atherosclerosis. Artif Cells Nanomed Biotechnol. 2019 May 22; 47(1): 2031–2041. <https://doi.org/10.1080/21691401.2019.1617724>.

التعبير الجيني ومستويات المثيلة لجين *PCSK9* للمرضى العراقيين المصابين بتصلب الشريان التاجي

مروة مظفر العطار¹ سلوى جابر العوادي² شيماء يوسف عبدالفتاح³

¹قسم علوم الحياة، كلية العلوم، الجامعة المستنصرية، بغداد، العراق.

²قسم تقنيات الوراثة الجزيئية والطبية، كلية التقنيات الاحيائية، جامعة النهريين، بغداد، العراق.

³مركز بحوث التقنيات الاحيائية، جامعة النهريين، بغداد، العراق.

الخلاصة:

يرتبط التعبير الجيني لجين *PCSK9* ارتباطاً وثيقاً بمستويات الدهون وخطر الإصابة بمرض تصلب الشرايين التاجية. تهدف الدراسة الحالية إلى قياس كمية التعبير الجيني للجين *PCSK9* وتأثير المثيلة على مستوى تعبيره الذي يساهم في التسبب في اضطراب الشريان التاجي الحاد. تضمنت الدراسة الحالية 150 شخصاً من المجتمع العراقي، 100 مريض بمرض الشريان التاجي و 50 من الأصحاء. تم تحديد تركيز *PCSK9* في كل عينات المصل المدروسة بواسطة تقنية ELISA، وتم تقدير مستويات التعبير عن جين *PCSK9* في الدم الكامل بطريقة qRT-PCR، واكتشاف مستوى مثيلة الحمض النووي في جين *PCSK9* باستخدام تقنية HRM-PCR. كان التعبير عن جين *PCSK9* أعلى في مجموعة مرضى *PCSK9* مقارنة بمجموعة الأصحاء 1.84 ± 6.06 في مجموعة مرضى *PCSK9* مقارنة بمجموعة التحكم 0.19 ± 1.00 . أيضاً كان تركيز بروتين *PCSK9* عالياً في مجموعة المرضى مقارنة بمجموعة الأصحاء $P = <0.01$. فضلاً عن ان مستويات مثيلة *PCSK9*، اظهرت فرقاً معنوياً بين مجموعتي الدراسة بمستوى $P=0.002$. في الختام، ترتبط مستويات بروتين *PCSK9* في المصل بمرض الشريان التاجي كما موضح في receiver operating characteristic (ROC) analysis، مما يؤدي إلى اعتبار أن تركيز بروتين *PCSK9* قد يكون مؤشراً جيداً لتطور مرض الشريان التاجي.

الكلمات المفتاحية: تصلب الشرايين التاجية، الاليزا، التعبير الجيني، المثيلة، *PCSK9*، RT-qPCR.