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# The Association of Single Nucleotide Polymorphism rs5883 in The CETP Gene with Oxidized-LDL Level in Coronary Atherosclerosis Patients

Noor K. Habash[1], Omar F. Abdul-Rasheed[2], and Mustafa A. Salman[3]

*1 Iraqi Center for Cancer and Medical Genetics Research, Mustansiriyah University, Baghdad, Iraq.*
*2 Department of Chemistry and Biochemistry, College of Medicine, Al-Nahrain University, Baghdad, Iraq.*
*3 Nu'man Teaching Hospital, Baghdad, Iraq.*

## Abstract

Atherosclerosis is a progressive inflammatory disorder of the arterial wall and can affect any artery in the body. When it occurs in the heart (Coronary atherosclerosis), it can cause MI, angina, and even sudden death; in the brain, stroke, and transient ischemic attack; and in the limbs causing claudication and critical limb ischemia. CETP plays a role in transferring the cholesterol from peripheral tissues to the liver through the collection of triglycerides of VLDL and LDL for exchange by the cholesteryl ester of HDL. Therefore, it has a role in HDL metabolism by converting it to LDL and increasing the risk of incident atherosclerosis.

Aim of the study: To investigate the rs5883 genotypes and their association with the coronary atherosclerosis and to study the impact of this SNP on the CETP gene with the lipid profile including the OX-LDL in patients and controls.

Patients and method: All patients underwent angiography to confirm diagnosis in a case- control study, comparing 60 volunteers as the control group. The lipid profile test was measured by colorimetric method, Ox-LDL was measured by ELISA technique and the CETP SNP genotypes by RT-PCR technique.

Results: There was a significant difference in genotypes in the rs5883 SNP, the heterozygous genotype (TC) was much more frequent in patients (20%) than controls (3.7%) with a significant difference (OR= 6.5, 95%CI=1.38-30.55). There was no association of this SNP with lipid profile, there was a significant impact of rs5883 with Ox-LDL, the medians of CT carriers significantly higher than CC genotypes.

***Keywords:*** *CETP, atherosclerosis, rs5883, Ox-LDL*

## Introduction

Coronary atherosclerosis is a progressive and chronic inflammatory disease (1). It is characterized by the formation and accumulation of atherosclerotic plaque and fatty substances inside the damaged arteries (2,3). The plaque is composed of low-density lipoprotein cholesterol (LDL-C) or cholesterol or even triglycerides and may have other substances existing in the blood that cause hardening and narrowing of the arteries. (4,5,6) .It is the main cause of coronary artery disease (CAD) (7). HDL-Cholesterol can induce atherosclerosis regression due to its anti-atherosclerotic action which can be reverse cholesterol transport (8). LDL particles are modified by non- oxidative alteration, oxidation, and glycooxidation. The oxidation of LDL plays a potent role in early stages of atherosclerosis, the association with increased release of oxygen free radicals and nitric oxide due to endothelial dysfunction, and reduced antioxidants such as vitamin A, glutathione, vitamin E and carotenoids (9). Environmental factors with multiple genetic factors or genetic variants have been evaluated to evaluate the relation with coronary atherosclerosis and its clinical manifestations or coronary artery disease (CAD) (10). 40% to 60% of coronary atherosclerosis risk factors are inherited and prevention strategies required defined knowledge of genetic risk factors (11). Accumulating evidence supports the role of genetic factors in the pathogenesis of the disease in addition to the family history and the hereditary grade is more than 50%. (12, 13) . CETP plays a role in the transfer of cholesterol from peripheral tissue to

**Corresponding Address:**
**Mustafa A. Salman**
*Nu'man Teaching Hospital, Baghdad, Iraq.*
**Email:** Mustafahajii79@yahoo.com

the liver by collecting triglycerides from VLDL and LDL for exchange with the HDL cholesteryl ester; therefore, it plays a role in HDL metabolism by converting it to LDL and increases the risk of atherosclerosis incident (14). The gene of CETP is a single gene located on chromosome 16 in the long arm consisting of 16 exons with 15 introns in the gene locus 16q12-16q21 and is about 25 kilo bases (kb) (15, 16).

Many mutations and SNPs in the CETP gene identified as a cause of CETP deficiency and impaired its function, but the association of these SNPs and susceptibility to atherosclerosis still lack consistency. (17, 18, 19). Furthermore, a single nucleotide polymorphism (SNP) could play a limited role in the genetic load of a particular disease in general (20). So understanding the level of genetic, environmental and lipids in the health state is of great interest (21). In addition to the relation of a particular SNPs and the susceptibility to coronary atherosclerosis has not been fully investigated and studied in Iraqi population. The study aimed to investigate the rs5883 genotypes and their association with coronary atherosclerosis and study the impact of this SNP on the CETP gene with the lipid profile, including OX-LDL in patients and controls.

## Methods

**Sample Collection**

A case-control study was carried out during the period from February 2019 to June 2020. It included 120 subjects, sixty subjects, (42 males, 18 females) with coronary atherosclerosis mean age ± SD (54.53 ± 8.48 years) and 60 subjects, (44 males, 16 females) healthy volunteers (controls) mean age ± SD (52.9 ± 8.86 years). All patients were investigated by cardiologists and underwent angiography examination for diagnosis the coronary atherosclerosis. The Institutional Review Board (IRB) of Al-Nahrain University, College of Medicine, Baghdad, Iraq, approved the research design decision No. 20190911 on 23/8/2021 and written consent was obtained for all patients included in the present study. The study excludes subjects with acute myocardial infraction, history of angina pectoris, congestive heart failure, cardiomyopathy, hypertension, diabetes mellitus, hyperlipidemia, or family history of hyperlipidemia. Blood samples were taken from the anti cubital vein and divided into two parts, part one: approximately five milliliters collected in a plain tube before angiography examination. The samples were left for 30 min. at room temperature. The tubes were then centrifuged at 3000 rpm for 15 minutes. The serum formed was divided into small aliquots and used for the immediate measuring of lipid profile by colorimetric assay and the rest was used to the measurement of Ox-LDL by ELISA technique. The second part of the blood samples: approximately two milliliters collected in EDTA tubes as whole blood and stored at (-20 C°) for the genetic measurements of CETP gene polymorphisms by the (RT-PCR) technique.

**Serum OX-LDL level**

The principle of measuring of Ox-LDL according to the commercially available kit (Bioassay, China) was a murine antibody specific for Oxidized-LDL has been pre-coated on to a 96 –well microplate with removable strips. Oxidized-LDL in standards and samples in competed by a biotinylated Oxidized- LDL sandwiched by the immobilized antibody and streptavidin-peroxidase conjugate. All unbound material is then washed away, and the peroxidase enzyme substrate is added. Color development is stopped, and the intensity of the color is measured. The unit was in ng/L.

Genetic analysis

The genomic DNA extraction was carried out by using the ReliaPrep™ Blood gDNA Miniprep System, Promega, USA. 20µl of proteinase K was used for each 1.5 micro centrifuge tube, then blood was added. 200 µl of cell lysis buffer was added and mixed for 10 sec. after incubation, 250 µl of binding buffer was added and mixed for 10 sec. by vortex, after washing step 100 µl of nuclease- free water was added. The RT-PCR was used to determine the CETP single nucleotide polymorphism genotypes. The following primer, farward 5`-TTAGCCTCCCTATACCCTTATT-3` and reverse 5`-GACCTAAGCCTGGTAGTTAAAG -3` was used to amplify the fragment of CETP gene as shown in table (1). To examine the optimum annealing temperature of primer, the DNA template was amplified with the same primer pair, (Forward) (Reverse), at annealing temperatures of 55, 58, 60, 63 and 65°C. PCR amplifications were performed with 20µl volumes containing 10µl GoTaq Green Master Mix (2X); 1µl for each primer (10pmol); 6µl nuclease free water and 2µl of template DNA. PCR cycling was performed with PCR Express (Thermal Cycler, Thermo Fisher Scientific, USA) with the following temperature program: denatured at 94oC for 4 min followed by 30 cycles of denaturation at 94°C for 30 sec.; annealing at 55, 58, 60, 63 or 65°C for 30 sec.; and extension at 72°C for 30 sec. A final extension incubation of 7 min at 72 ° C was included, followed by a 10 min incubation at 4 ° C to stop the reactions. Table (1).

Table (1): The primer optimization

| Primer name | Sequence | Annealing temperature (C) | Product size (bp) |
|---|---|---|---|
| CEPT-F | `TTAGCCTCCCTATACCCTTATT3`5 | 60 | 808 |
| CETP-R | `GACCTAAGCCTGGTAGTTAAG3`5 | 60 | 808 |

**PCR cycles**

The PCR cycle was performed as shown in table (2). The PCR product was determined by running the product on agarose gel electrophoresis, ethidium bromide (10mg / ml) was used, and the stained bands in gel were visualized using a gel image system.

Table (2): The PCR cycling

| Steps | ℃ | m:s | Cycle |
|---|---|---|---|
| Initial denaturation | 95 | 05:00 | 1 |
| Denaturation | 95 | 00:30 | 30 |
| Annealing | 60 | 00:30 | 30 |
| Extension | 72 | 00:30 | 30 |
| Final extension | 72 | 07:00 | 30 |
| Hold | 10 | 10:00 | 1 |

**The PCR Optimization**

The PCR product was determined by running the product on agarose gel electrophoresis, ethidium bromide (10mg / ml) was used, and the stained bands in gel were visualized using a gel image system. Figure (1)

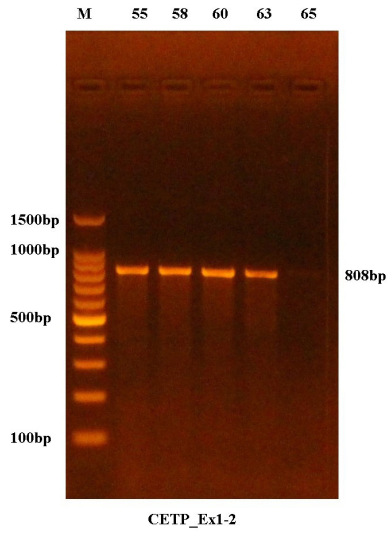


Figure-1, The PCR product visualized using agarose gel electrophoresis.

SPSS software version 25 (SPSS, Chicago) was used for statistical analyses. Categorical variables were analyzed by Chi-square test. Data with normally distribution were presented as mean ± standard deviation and analyzed with one-way ANOVA. Data with non-normal distribution were presented as median and range and analyzed with Mann Whitney U test (for two groups comparison) or Kruskal Wallis (for three groups comparison). The chi-square was used for testing the deviation from the Hardy-Weinberg equilibrium (HWE). (22)

## results

Table (3) shows the lipid profile in patients and controls. Data regarding the components of lipid profile were found to be non-normally distributed. Accordingly, the nonparametric Mann Whitney U test was used to compare the medians between the two groups. Median serum level of TC, TG and non-HDL-C in patients were 189 mg/dl, 153 mg/dl and 150 mg/dl, respectively compared with 118 mg/dl, 88.5 mg/dl and 55.35 mg/dl, respectively in controls with highly significant differences. Likewise, the atherogenic index was significantly higher in patients than in controls (0.66 versus 0.21). In contrast, median serum level of HDL in controls was 55.4 mg/dl which was significantly higher than that of patients (32 mg/dl), LDL was differs significantly in patients than controls (86 mg/dl versus 76) as shown in table (3).

Table (3): Lipid profile indices in different groups

| Variables | Patients (n=60) | Controls (n=60) | P- Value |
|---|---|---|---|
| **TC, mg/dl** | | | |
| Mean±SD | 179.67±49.1 | 116.04±31.95 | **0.000>** |
| Median | 189.0 | 118.0 | |
| **TG, mg/dl** | | | |
| Mean±SD | 157.62±61.26 | 94.51±54.96 | **0.000>** |
| Median | 153.0 | 88.5 | |
| **HDL, mg/dl** | | | |
| Mean±SD | 30.49±6.92 | 59.55±26.7 | **0.000>** |
| Median | 32.0 | 55.4 | |
| **LDL, mg/dl** | | | |
| Mean±SD | 102.53±41.12 | 76.61±34.35 | **0.004** |
| Median | 86.0 | 76.0 | |
| **VLDL, mg/dl** | | | |
| Mean±SD | 28.71±12.1 | 26.37±7.81 | 0.925 |
| Median | 22.95 | 22.9 | |
| **Atherogenic index** | | | |
| Mean±SD | 0.63±0.24 | 0.27±03 | **0.002>** |
| Median | 0.66 | 0.21 | |

TC: total cholesterol, TG: triglycerides, HDL: high density lipoprotein, LDL: low density lipoprotein, VLDL: very low density lipoprotein.

**Serum level of OX-LDL**

The median serum level of oxidized LDL in the patients was 2269.43 ng/L (range 1191.06 -2881.6 ng/L) which was very close to that of the controls (median= 2263.29 ng/L, range 1496.56- 4564.92 ng/L) with no significant difference. Figure (2).

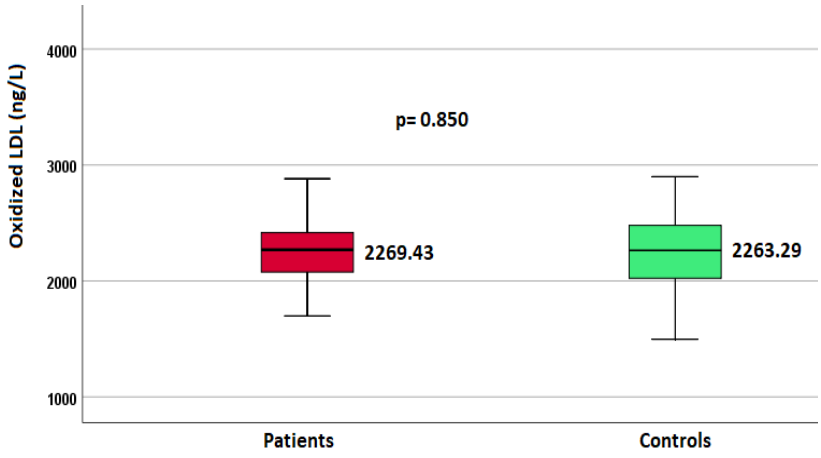


Figure-2, Serum levels of oxidized LDL. The median serum level of oxidized LDL in the patients was 2269.43 ng/L (range 1191.06 -2881.6 ng/L) which was very close to that of the controls (median= 2263.29 ng/L, range 1496.56- 4564.92 ng/L) with no significant differences.

**The sequence of rs5883 single nucleotide polymorphism**

The heterozygous genotype (TC) was far more frequent in patients (20%) than controls (3.7%) with a significant difference (OR= 6.5, 95%CI=1.38-30.55, p=0.018). At the allelic level, the mutant allele (T) was more frequent in patients than in controls (10% versus 1.85%) with a significant difference (OR=5.89, 95%CI= 1.29- 26.94, p= 0.022) as shown in table (4).

Table (4): The frequency of different genotypes and alleles of the CETP polymorphism SNP rs5883 in patients and controls

| rs5883 | Patients n= (60) | Controls n=(54) | *P*-value | OR (95%CI) |
|---|---|---|---|---|
| **Genotypes**<br>CC<br>TC<br>HWE | <br>(80%)48<br>(20%)12<br>0.389 | <br>(96.30%)52<br>(3.70%)2<br>0.888 | 0.018 | 1.0<br>(1.38-30.55)6.5 |
| **Alleles**<br>C<br>T | (90%)108<br>(10%)12 | (98.15%)106<br>(1.85%)2 | 0.022 | 1.0<br>(1.29-26.94)5.89 |

**Association of rs5883 SNP in CEPT gene with Lipid Profile and Ox-LDL in Patients and Controls.**

In patients' group, there was a significant association of this SNP with Ox-LDL, the medians of CT carriers significantly higher than CC genotypes. The medians of the atherogenic index related to CT genotypes were also significantly higher than CC. P=0.006. Table (5).

Table (5): The association of rs5883 genotypes with lipid profile, OX-LDL in patients and controls.

| Variables | Patients | | p-value | Controls | | P-value |
|---|---|---|---|---|---|---|
| | CC(n=48) | CT(n=12) | | CC | CT | |
| TC, mg/dl | 190.5<br>(116-298) | 131<br>(116-229) | 0.129 | 118.5<br>(63.9-210) | 65<br>(65-65) | **0.028** |
| TG, mg/dl | 126<br>(77-306) | 166.5<br>(115-212) | 0.103 | 83.5<br>(42-360) | 89<br>(89-89) | 0.647 |
| HDL, mg/dl | 32.5<br>(21-43.6) | 29<br>(22.5-36.6) | 0.355 | 60.2<br>(23-116.1) | 65.9<br>(65.9-65.9) | 0.783 |
| LDL, mg/dl | 86<br>(47.7-198) | 107<br>(59.5-161.2) | 0.711 | 76<br>(20.1-198) | 54.9<br>(54.9-54.9) | 0.142 |
| VLDL, mg/dl | 22.85<br>(15.4-71.9) | 27.5<br>(18.7-38) | 0.824 | 22.6<br>(15.4-44.8) | 32.1<br>(32.1-32.1) | 0.099 |
| Atherogenic index | 0.63<br>(0.02-1.16) | 0.78<br>(0.5-0.95) | **0.006** | 0.16<br>(0-1.46) | 0.13<br>(0.13-0.13) | 0.842 |
| Non-HDL-c, mg/dl | 154.1<br>(57-714.6) | 103.7<br>(80.4-273) | 0.395 | 66<br>(12-122.4) | 99.7<br>(99.7-99.7) | 0.067 |
| Oxidized LDL | 2232.3<br>(1191-2599.9) | 2636.4<br>(1920.8-2881.6) | **0.001** | 2263<br>(1496-3645) | 2356<br>(2356-2356) | 0.521 |

## Discussion

Taking into account the central role of CETP in lipid metabolism, the present study investigated the association of rs5883 SNP with this gene and estimated the risk of coronary atherosclerosis in Iraqi populations. The association of this SNP with lipid profile in general in addition to a novel association studied in the present study of this SNP with OX-LDL serum level in patients of coronary atherosclerosis.

First, the serum level of OX-LDL in patients and controls was very close with no significant differences. The results of

statistical analysis for rs5883 genotypes showed that, there was a significant difference genotypes in rs5883 SNP, (TC) genotype was more frequent in patients (20%) than control group (3.7%) with a (OR=6.5, 95%CI= 1.38-30.55, P=0.018). This result is in agreement with the results obtained by (Kumar et al., 2014) (23). Who found a strong association with coronary atherosclerosis risk among the cases versus controls in South Indians. In contrast to a study by (Arikan et al., 2019) (14) which concluded that there was a lack of association related to rs5883 SNP in patients with atherosclerosis comparable to the control group in his study. The main objective of the examination was to assess the polymorphism of the CETP gene and its effect on serum lipid levels and OX-LDL in Iraqi subjects with atherosclerosis. This study selects the CETP rs5883 polymorphism since some studies discussed its relationships with the HDL level, but no previous study examined their relationships with other lipid disorders and in light of the fact, there was no Iraqi examination study on this polymorphism of the CETP gene. In addition to the novel assessment of this SNP and its influence on oxidized LDL concentration in serum.

To date, there have been no previous studies on the impact of the CETP genetic SNP rs5883 polymorphism on OX-LDL levels in patients with atherosclerosis. In this study, the influence of rs5883 polymorphism genotypes with the lipid profile in patients of coronary atherosclerosis was non-significant including the level of HDL-C. The negative influence of rs5883 SNP with HDL-C agree with study of (Wang J. et al. 2013) (24) who didn't find significant impact of rs5883 SNP genotypes and the level of HDL-C, and with results obtained by (Arikan, G. D. et al., 2019) (14). Who conclude that there was lack of impact of the rs5883 in the CEPT gene with the HDL-C level. The present study found a novel association of this SNP genotypes with OX-LDL concentrations in serum of patients of coronary atherosclerosis. CC genotype significantly lower than CT genotype. The mechanism which links CEPT gene genetic polymorphisms to coronary atherosclerosis are widely unclear but the linkage of CETP polymorphisms and serum OX-LDL levels may explained a possible mechanism that may need further investigations in addition to the limitations of this study related to the population sample size and ethnic diversity which hampered the ability to detects some significant associations.

**Conclusions:**

SNP (rs5883) variations in CETP gene is associated with the patients of coronary atherosclerosis. Rs5883 SNP genotype is significantly associated with the serum level of OX-LDL in patients, CT carriers significantly higher than CC genotypes.

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**Conflict of interest:**

there was no Conflict of interest to be declared by the authors.

**Authors' contribution:**

The first author writes the paper and analyzed the result statistically and co-suggest the study, the second author suggests the study and co-analyzed the result, the third author collects the samples, all the authors read and approved the final draft.

**Ethical approval:**

The Institutional Review Board (IRB) of Al-Nahrain University, College of Medicine, Baghdad, Iraq, approved the research, Decision No. 20190911 on 23/8/2021, and written consent was obtained for all patients included in the present study.

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