Methylenetetrahydrofolate reductase C677T gene polymorphism and the association with dyslipidemia in type 2 diabetic Palestinian patients

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Abstract

Background: Dyslipidemia in diabetes is common and characterized by hypertriglyceridermia with decreased levels of high-density lipoprotein. The objective of this study was to assess the prevalence of MTHFR C677T polymorphism in Palestinian T2DM patients and to investigate the association between this polymorphism and lipid profile in diabetic patients with and without dyslipidemia.

Methods: A total of 208 T2DM patients including 98 with dyslipidemia and 110 without dyslipidemia were enrolled in this study. The MTHFR C677T genotyping was conducted by PCR-RFLP followed by agarose gel electrophoresis.

Results: There were no significant differences in either the genotype distribution or allele frequency in T2DM patients with or without dyslipidemia (37.8% CC, 54% CT, 8.2% TT vs. 48.2% CC, 41.8% CT, 11% TT; p = 0.209). However, among the dyslipidemic group, the TT carriers have a higher HDL level (46.8 ± 17.8) compared to (CC+CT) carriers (34.68 ± 11.9) (p = 0.01). In the group without dyslipidemia, there was a significant elevation in diastolic blood pressure (DBP) among the CC carriers (83.6 ± 10.6) compared to those who carried at least one mutant allele (CT+TT) (78.1 ± 11.1) (p = 0.009).

Conclusions: The study shows that in our Palestinian population the MTHFR 677TT genotype lowers DBP significantly in patients without dyslipidemia and is related to increased level of HDL in diabetic dyslipidemia patients.

KEYWORDS
C677T SNP, dyslipidemia, lipid profile, MTHFR, T2DM
INTRODUCTION

Type 2 diabetes mellitus (T2DM) is the most prevalent type of diabetes in adults, often associated with overweight and obesity and results in insulin resistance when there are insufficient insulin production and failure of the body to respond appropriately to insulin due to hyperglycemia. It is a multifactorial disorder that determined by several genetic and environmental factors and thus, predicting the probability of T2DM is significant and is beneficial in early diagnosis and evading serious complications. The clinical background of T2DM can be unremarkable or mild for many years. Consequently, late complications such as retinopathy, nephropathy, neuropathy, acute myocardial infarction, stroke, atherosclerosis, and serious infections can occur. In the last decades, the prevalence of T2MD has been rising across the globe, particularly in low- and middle-income regions.

In 2019, there were 174.3 per 1,000 Palestinians (aged 20–79 years) complaining of diabetes with a prevalence of 6.7%. The Palestinian annual health report of 2019 declared that the total number of new diabetic cases was 5671 with an incidence of 210.4 per 100,000. Furthermore, diabetes and its related complications form the third cause of death with 12.1% of all mortalities in Palestine following the cardiovascular diseases (29.9%) and cancer (15.5%). A study conducted by Diabetes Care Center at Augusta Victoria Hospital, Jerusalem, reported that among 1308 diabetic patients: hypertension and dyslipidemia were found in 23% and 37.3% of the patients, respectively. Moreover, 16.3% of them had a previous history of the macrovascular disease (myocardial infarction or stroke), and 25.9% had microvascular complications.

Methylenetetrahydrofolate reductase (MTHFR) enzyme, that involved in folate metabolism, reduces 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolates which is required as a co-substrate in the conversion of homocysteine to methionine. A single nucleotide polymorphism (SNP) rs1801133 or MTHFR C677T (C to T transition polymorphism) was described in the MTHFR gene which is located on chromosome 1p36.3 and encompasses 2.3kb of DNA, this SNP causes limited activity of the MTHFR enzyme due to the amino acid change from alanine to valine (A222V); hence, any impairment in this process will increase the circulating homocysteine (Hcy) levels.

Methylenetetrahydrofolate reductase C677T polymorphism is associated with several diseases, including cardiovascular events, common malignancies like lung and breast cancers, and infertility. The association between this SNP and T2DM has a universal variety, a systematic review including 4855 participants with T2DM and 5242 controls descending from diverse ethnicities showed no association between MTHFR C677T genotypes and risk of T2DM. However, a recently emerged meta-analysis indicated that MTHFR C677T polymorphism was significantly linked to T2DM, especially in Asian populations.

Type 2 diabetes mellitus is considered a secondary cause of dyslipidemia and untreated diabetic patients can suffer from poor management response which leads to hyperlipidemia-related complications, the most serious are cardiovascular ones. People with T2DM may have different types of dyslipidemias. The characteristic features of diabetic dyslipidemia are a high plasma triglyceride (TG) concentration, low concentration of high-density lipoprotein (HDL) cholesterol, and increased concentration of low-density lipoprotein (LDL) cholesterol. Changes increase the risk of cardiovascular events among diabetic patients, some evidence revealed that diabetes history play as same as the history of myocardial infarction in cardiovascular disease (CVD) development and mortality.

Insulin resistance has a key role in the pathogenesis of diabetic dyslipidemia, the chief cause of the three features of diabetic dyslipidemia is the increased free fatty-acid release from insulin-resistant fat cells, several circumstances are reasonable for diabetic dyslipidemia: insulin effects on liver apoprotein production, regulation of lipoprotein lipase, actions of cholesteryl ester transfer protein, and peripheral actions of insulin on adipose and muscles.

Homocysteine is an independent risk factor for CVD development by increasing the risk associated with all lipid measures which have been associated with damage to the lining of arteries and atherosclerosis. As serum lipids are reported to be affected by MTHFR gene polymorphisms, we hypothesized that MTHFR C677T may affect serum lipid profiles in diabetic patients and thus predisposing them to the risk of dyslipidemia. Therefore, the study aim was to assess the prevalence of MTHFR C677T polymorphism among T2DM Palestinians and to evaluate the possible association with diabetic dyslipidemia.

METHODS

2.1 Study participants

A case control study was carried out from January to April 2019. A total of 208 T2DM patients aged >50 years were recruited from Palestine Medical complex (Ramallah, Palestine) hospital, all demographic and clinical data including age, gender, body mass index (BMI), treatment, and diabetic complications were taken from their medical records. All biochemical measurements including fasting plasma glucose (FPG), Hemoglobin A1c (HbA1c), total cholesterol (TC), TG, HDL cholesterol were performed during the hospital admission examination. LDL cholesterol was calculated using the Friedewald formula. Blood pressure was measured in sitting position, on the left arm, after a 5-min rest by a nurse, with a mercury sphygmomanometer. T2DM was defined according to the WHO criteria: fasting plasma glucose (FPG) ≥ 126 mg/dl and/or currently being treated with medication for diabetes. Dyslipidemia was defined by: TC level ≥ 240 mg/dl, and/or TG level ≥ 150 mg/dl, LDL cholesterol level ≥ 140 mg/dl, HDL cholesterol level < 40 mg/dl, and/or the use of a lipid-lowering drug. Accordingly, the studied subjects were stratified into two groups: T2DM patients with dyslipidemia who fulfilled diabetes and dyslipidemia diagnostic criteria as described above, T2DM patients without dyslipidemia (as controls) who diagnosed
with diabetes; their FPG ≥126 mg/dl, fasting TC <200 mg/dl and TG <150 mg/dl, and have been never treated with lipid-lowering agents.

Written informed consent was obtained from all enrolled participants. The study protocol was approved by Al-Quds University Research Ethics Committee (71/REC/2019). The work has been carried out in accordance with the code of Ethics of the World Medical association (Declaration of Helsinki) for experiments in humans.

### 2.2 Blood samples and DNA extraction

Blood samples (5 ml) were collected in tubes containing 0.5 ml of ethylenediamine tetra-acetic acid (EDTA) as an anticoagulant. Genomic DNA was extracted from whole blood (200 μl) using a genomic QIAamp DNA purification kit according to the manufacturer’s instructions (Qiagen, Hilden). The DNA concentration was measured by a NanoDrop 1000 spectrophotometer (Thermo Fisher). DNA samples were frozen at −20°C until processed.

### 2.3 PCR-restriction fragment length polymorphism (PCR-RFLP)

For *MTHFR* genotyping, DNA samples were amplified by polymerase chain reaction (PCR) using the forward primer 5’ TTTGAGGCTGACCTGAAGCCTTGAGGAG 3’, and the reverse primer 5’GAGTGGTAGCCCTGGATGGGAAAGATCCCG as previously described. Briefly, the reaction was carried out using 3 μl of the extracted DNA in a final volume of 25 μl, which contained 12.5 μl PCR BIO HS Taq Mix Red, 8.5 μl double distilled water (dH2O), 0.5 μl of each primer (10 pmol/μl). The amplification condition was as followed: initial denaturation at 95°C for 5 min followed by 32 cycles of 95°C for 30 s, 65°C for 30 s, 72°C for 40 s. The final extension was carried out at 72°C for 6 min. The PCR product was confirmed using 2% agarose gel stained with 0.8 μl ethidium bromide (10 mg/ml). The amplified PCR product was digested with 1 μl of HinfI endonuclease enzyme and incubated for 2 h at 37°C. The final genotype patterns were determined and the product was seen on a 2% agarose gel stained by ethidium bromide and visualized by UVITEC Gel Documentation System. A 10% blind random sample was re-amplified and digested (using the same conditions as described above) to confirm the genotyping results.

### 2.4 Statistical analysis

The genotype frequencies were tested for Hardy–Weinberg equilibrium by calculating a chi-square statistic and corresponding p-value. Pearson’s Chi-square analysis was performed to test allele and genotype frequency differences between the two studied groups (with and without dyslipidemia). ANOVA was used to assess the association between *MTHFR* genotypes and continuous variables. Logistic regression was used to measure odd ratio (OR) for diabetic dyslipidemia adjusted for age, gender, and BMI. p-value less than 0.05 was considered significant. Analysis was performed using SPSS program version 23.

### 3 RESULTS

#### 3.1 Demographic and Biochemical characteristics of the study participants

This case control study includes 208 T2DM patients. Among them, 47% were diagnosed with diabetic dyslipidemia, and 53% without dyslipidemia. Of all subjects, 65.4% of them (n = 136) have diabetic complications (38.9% CVD, 15.9% nephropathy (DN), 14.4% retinopathy (DR), and 2.9% with diabetic foot). The gender distribution, mean age, clinical, and biochemical parameters of T2DM patients with and without dyslipidemia are shown in Table 1. The mean BMI was higher in T2DM patients with dyslipidemia (30.0 ± 3.7) compared to those without dyslipidemia (24.8 ± 3.7) (p < 0.05). The lipid profiles including TC, TG, HDL, and LDL were also statically different in T2DM with dyslipidemia compared to those without dyslipidemia (p < 0.05). The mean FPG was higher within the dyslipidemic group but without any significant difference. The means of age and blood pressure measurements were also not significantly different between the two groups.

#### 3.2 Genotyping of *MTHFR* C677T variant

Methylenetetrahydrofolate reductase C677T genotyping was performed by PCR followed by RFLP. The PCR product revealed a band

| TABLE 1 Demographic, clinical, and biochemical parameters of T2DM with and without dyslipidemia |
|---------------------------------|---------------------------------|------------------|---|
|                                  | DM with dyslipidemia | DM without dyslipidemia | p-Value |
| Number (n)                       | 98                 | 110               |   |
| Gender (M/F)                     | 65/33              | 65/45             |   |
| Age (years)                      | 62 ± 9.6           | 63.4 ± 10.7       | 0.324 |
| BMI (kg/m²)                      | 30 ± 3.7           | 24.8 ± 3.7        | 0.0 |
| BP-sys (mmHg)                    | 135.6 ± 19.1       | 139.6 ± 20.4      | 0.146 |
| BP-dias (mmHg)                   | 78.1 ± 13          | 80.8 ± 11.2       | 0.117 |
| FPG (mg/dl)                      | 240.7 ± 107.9      | 229.9 ± 95.6      | 0.444 |
| HbA1c                            | 8 ± 1.3            | 8.2 ± 1.5         | 0.584 |
| TG(mg/dl)                        | 254.9 ± 149.1      | 149 ± 60          | 0   |
| TC(mg/dl)                        | 236.5 ± 86.6       | 161.6 ± 50.3      | 0   |
| HDL(mg/dl)                       | 35.7 ± 12.9        | 49.5 ± 18         | 0   |
| LDL(mg/dl)                       | 162.1 ± 54.7       | 97 ± 42.3         | 0   |

Abbreviations: BMI, body mass index; BP-dias, diastolic blood pressure; BP-sys, systolic blood pressure; FPG, fasting plasma glucose; HbA1c, glycated hemoglobin; HDL, high-density lipoproteins; LDL, low-density lipoproteins; TC, total cholesterol; TG, triglyceride.
of 173 bp as shown in Figure 1A. The genotypes were determined based on the banding patterns of the digested PCR products. The wild-type genotype (CC) was identified by the presence of one band of 173 bp, the mutant genotype (TT) was identified by the presence of two bands of 125 bp and 48 bp and the heterozygous genotype (CT) was identified by the presence of three bands of 173, 125, and 48 bp. Figure 1B showed representative samples including the three genotyping patterns. The genotyping distribution was in Hardy–Weinberg equilibrium in both groups with and without dyslipidemia (p > 0.05). The Allele and genotype distributions in the studied groups are shown in Table 2. The most frequent genotype in the dyslipidemic group was CT in more than half of the cases (54%), followed by CC (37.8%) and TT (8.2%). For those without dyslipidemia: CC, CT, and TT genotypes were 48.2%, 41.8%, and 10%, respectively. Despite these variations, there were no significant differences in the genotype and allele frequencies between the two study groups (p > 0.05). The association between the genotypes and dyslipidemia was tested by multivariable logistic regression analysis using three genetic models: dominant (CC vs. CT+TT), recessive TT vs. (CC+CT) and over-dominant CT vs. (CC+TT) adjusted for age, gender, and BMI. No association between MTHFR C677T genotypes and risk of dyslipidemia was observed under these genetic models (p > 0.05) (data not shown).

3.3 | Association of MTHFR C677T variant with clinical and biochemical parameters

Because of potential confounding between diabetic dyslipidemia and increased TG level, all biochemical and clinical data were stratified by lipidemic status across the MTHFR genotypes (Table 3). In T2DM group without dyslipidemia, there was a significant difference in DBP among the different genotypes (p = 0.034) (Table 3). The CC carriers showed a significant elevation in DBP (83.6 ± 10.6) compared to those who carried at least one mutant allele (CT+TT) (78.1 ± 11.1) (p = 0.009). Moreover, Kruskal–Wallis H test showed a statistically significant difference in DBP between the studied groups (CC vs. CT+TT), χ² = 7.051, p = 0.008, with a mean rank DBP of 63.86 for CC genotype and 47.73 for (CT+TT) genotypes. Likewise, the systolic blood pressure was higher in the CC group but it was not statistically significant (p = 0.75) (Table 3). Among T2DM cases with dyslipidemia, the mean HDL was significantly different between the different genotypes (p = 0.038) (Table 3), further analysis revealed that the TT carriers had higher HDL (46.8 ± 17.8) compared to (CC+CT) carriers (34.68 ± 11.9) (p = 0.01). Kruskal–Wallis H test showed a statistically significant difference in HDL between the two groups (TT vs. CC+CT) (p = 0.037) with a mean rank HDL of 69.6 for TT carriers and 47.7 for (CC+CT) carriers. However, no association was found between the MTHFR genotype and age, BMI, TC, TG, as well as HbA1C in both groups (p > 0.05) (Table 3).

4 | DISCUSSION

Type 2 diabetes mellitus is a combined genetic and environmental disorder that affects more than 90% of patients with diabetes.1–4 In many diabetic patients, a particular type of dyslipidemia called diabetic dyslipidemia consists of low HDL and increased TG levels, the pattern most commonly seen in T2DM and may be a treatable risk factor for subsequent cardiovascular disease by understanding its pathophysiology.20

People from different ethnic groups had different genetic susceptibility to T2DM.24 MTHFR C677T is one of the most frequently studied mutations among diabetics. MTHFR enzyme have a key role in homocysteine and folate metabolism, it is shown that homozgyous (TT) and heterozygous (CT) genotypes reduce the enzyme activity by 70% and 35%, respectively, compared to the wild type CC genotype and thus may associated with increased plasma homocysteine levels and with T2DM or its complications.16,17

The frequency of 677TT genotype is highly variable among the diabetic population. Our study showed that the overall frequency of 677TT genotype was 9.1% in all diabetic subjects, which is lower than that in Egypt (32.5%)22 and Israel (18%), but it is in agreement with some countries in the region such as Iran 7%27 and Turkey 7%.28 In contrast, other studies reported a significantly lower frequency of TT genotypes in T2DM patients from India (1%) and United Arab Emirates (3%).29,30
TABLE 2 Genotype and allele frequencies of MTHFR C677T polymorphism in T2DM patients with and without dyslipidemia

| Genotypes | T2DM with dyslipidemia n(%) | T2DM without dyslipidemia n(%) | p-Value |
|-----------|-----------------------------|-------------------------------|---------|
| CC        | 37 (37.8%)                  | 53 (48.2%)                    | 0.209   |
| CT        | 53 (54%)                    | 46 (41.8%)                    |         |
| TT        | 8 (8.2%)                    | 11 (10%)                      |         |
| Allele    |                             |                               |         |
| C         | 127 (64.8%)                 | 152 (69%)                     | 0.352   |
| T         | 69 (35.2%)                  | 68 (31%)                      |         |

Studying the associations of MTHFR C677T polymorphism and T2DM was performed previously but inconsistent results have been reported. In this study, the genotype distribution was also studied in a group of unrelated non-diabetic individuals (n = 93), the mean age (years ± SD), the mean BMI and the mean FPG (mg/dl ± SD) were 45 ± 9.0, 29.3 ± 5.90 and 86.5 ± 8, respectively. There was no significant difference in MTHFR C677T genotype frequencies between the diabetic and non-diabetic group (43.3% CC, 47.6% CT, 9.1% TT vs 34.4% CC, 58.1% CT, 7.5% TT; p = 0.24) (data not shown). However, due to the absence of complete lipid profile for this non-diabetic group, we did not include them in any further analysis.

In this study, the association between MTHFR C677T polymorphism and dyslipidemia has been investigated in 208 T2DM Palestinian patients with and without dyslipidemia. As expected, the group with dyslipidemia had higher TC, TG, LDL levels, and lower HDL (p < 0.05) which probably contribute to accelerated atherosclerosis. The genotyping results showed no differences in the genotype distribution and allele frequency between T2DM individuals with and without dyslipidemia. Moreover, logistic regression models adjusted for age, gender, and BMI revealed no evidence for association with risk of dyslipidemia in three genotypic models. However, when the analysis was stratified by lipidemic status across the MTHFR genotype, the HDL level was statistically higher amongst the TT carrier compared to CC+CT carriers in dyslipidemia group. Interestingly, a protective effect of the T allele among T2DM patients with CAD was observed by Kucukhuseyin et al. who showed that individuals with CC genotype have higher level of LDL and TG compared to TT and CT individuals. Inconsistent to our results, Liu et al confirmed the association of MTHFR C677T with dyslipidemia risk in patients with mild-to-moderate essential hypertension.

Our findings indicated that, among the T2DM without dyslipidemia, the CC carriers have a significant high DBP compared to the TT carries. Likewise, the T allele carriers among Turkish patients with diabetic and non-diabetic coronary heart disease showed lower levels of DBP. Such findings were also found in the Japanese population showing that C677T mutation was associated with lower blood pressure, which was protective for cerebral vascular disease.

However, an observational data revealed that MTHFRRTT genotype in 18–70 year old adults was associated with an increased risk of hypertension (systolic BP ≥ 140 and/or a diastolic BP ≥ 90 mmHg).

In addition, it was noted that the MTHFR C677T polymorphism significantly increased the risk of hypertension in rural Indonesian-Sudanese population.

On the other hand, although 65% of the studied subjects have diabetic complications, we could not find any association between the MTHFR C677T genotype distribution and the prevalence of CVD, DN and DR among the studied population (p > 0.05). This is consistent with a study findings showed that MTHFR C677T polymorphism is not a risk factor for diabetic complications or even diabetes in the south Indian population. Also, several studies reported no association between this SNP and coronary artery disease or nephropathy in T2DM patients. In contrast, other studies showed that cases of diabetic nephropathy have a significantly higher frequency of the mutant genotype MTHFR 677 TT. As well, a significant association between MTHFR C677T polymorphism and vascular complications of T2DM was found when comparing 1984 diabetic patients with vascular complications to 1703 T2DM without vascular complications. We believe that lifestyle; glucose control and compliance with statin therapy may affect the lipid profile and reduce vascular complications in diabetic patients.

Given that the association studies were conducted in different population and thus conflicting results of investigations of this mutation have been reported. It was obvious that these variations were related to ethnicity and other intervening variables such as folate, vitamin B12, and homocysteine levels that must be considered in genotype–phenotype correlation studies.

A study conducted in Israel showed no significant differences of MTHFR genotype distribution for both A1298C and C677T polymorphisms in patients with or without DN. In that study, stratification analysis based on serum folate levels showed a lower incidence of DN in 1298 CC individuals suggesting that the homozygous state may have a protective effect against DN. However, all individuals who had the 1298 CC genotype also had the 677CC wild-type genotype. Thus, the protective effect of the studied polymorphism may be mediated by the absence of a deleterious polymorphism within the same gene. Interestingly, it has been reported that the role of MTHFR genotypes can be changed by different dietary intake. A study conducted in India showed that vegetarian people had higher homocysteine levels irrespective of the MTHFR genotype. Furthermore, it is reported that folate supplementation can prevent the increase of homocysteine levels and overcome the reduction of MTHFR enzyme activity associated with the mutant enzyme.

Moreover, it is important to not ignore the synergistic effect of other polymorphisms in other genes that involved in homocysteine metabolism. Several studies reported that MTHFR 667TT and methionine synthase reductase (MTRR) 66GG genotypes showed higher serum Hcy levels associated with higher serum TG and TC levels in hypertensive or diabetic patients. Thus, a functional study for the role of MTHFR C677T polymorphism in diabetic dyslipidemia in the Palestinian population is a subject for further analysis. Although the study population is inclusive of both patients with and without dyslipidemia, this is a single-center study and thus generalizability of these results should take into account the small sample size and thereby decreased statistical power and the
TABLE 3 Subject characteristics by MTHFR C677T genotype in T2DM patients with and without dyslipidemia

| Genotypes | T2DM with dyslipidemia (n = 98) | T2DM without dyslipidemia (n = 110) | p-Value |
|-----------|-------------------------------|------------------------------------|---------|
|           | CC (n = 37)                  | CT (n = 53)                       | TT (n = 8)                      | CC (n = 53) | CT (n = 46) | TT (n = 11) | p-Value |
| Age       | 62.2 ± 10                     | 62.2 ± 9.7                        | 59.3 ± 7.7                      | 0.708       | 62.4 ± 12   | 64.1 ± 9.5  | 65 ± 9.1  | 0.634     |
| BMI       | 29.8 ± 4                      | 29.9 ± 3.6                        | 31.2 ± 2.8                      | 0.631       | 24 ± 3.1    | 25.4 ± 4.05 | 25.8 ± 3.7 | 0.089     |
| BP-sys    | 135.6 ± 20.1                  | 136.1 ± 17.1                      | 132.4 ± 28.2                    | 0.876       | 141 ± 23    | 138.8 ± 16.6 | 137.8 ± 22.2 | 0.750     |
| BP-dias   | 76.8 ± 13.5                   | 79.3 ± 13.4                       | 76.3 ± 8.7                      | 0.629       | 83.6 ± 10.6 | 78.1 ± 11   | 78.2 ± 12.1 | 0.034*    |
| FPG       | 237 ± 100                     | 244.7 ± 110.8                     | 231.5 ± 134.9                   | 0.917       | 215.4 ± 89  | 241 ± 100.6 | 253.3 ± 103.5 | 0.293     |
| HbA1c     | 8.2 ± 1.3                     | 79 ± 1.2                          | 79 ± 2                          | 0.546       | 8 ± 1.4     | 8.2 ± 1.7   | 8.1 ± 1.3  | 0.924     |
| TG        | 274.6 ± 200.8                 | 267.3 ± 111                       | 213.6 ± 70.8                    | 0.503       | 148 ± 63    | 148.3 ± 52.6 | 155.8 ± 76.7 | 0.924     |
| TC        | 248.9 ± 101.2                 | 228.2 ± 79.9                      | 233.7 ± 49.7                    | 0.536       | 158.5 ± 54.7 | 165.1 ± 46.2 | 161 ± 48.4 | 0.806     |
| HDL       | 34.6 ± 13.1                   | 34.7 ± 11.3                       | 46.8 ± 17.8                     | 0.038*      | 50.1 ± 18.4 | 48.5 ± 18  | 50.2 ± 18.6 | 0.907     |
| LDL       | 1599 ± 45.9                   | 1612 ± 60.5                       | 1794 ± 55.5                     | 0.65        | 96 ± 47     | 99.6 ± 37.7 | 90.8 ± 39.8 | 0.807     |

*p < 0.05 was considered significant (obtained by ANOVA). Data are presented as mean ± SD.
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