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***In vivo* triglyceride synthesis in subcutaneous adipose tissue of humans correlates with plasma HDL parameters**

Demidmaa Tuvdendorj, MD, PhD¹, Alejandro O. Munoz, MD¹, Viviana Ruiz-Barros², Jean-Marc Schwarz, PhD², Giuseppe Montalto, MD³, Manisha Chandalia, MD¹, Lawrence C. Sowers, PhD⁴, Manfredi Rizzo, MD, PhD^{3,4}, Elizabeth J Murphy, MD, DPhil², and Nicola Abate, MD¹

¹Departments of Internal Medicine, University of Texas Medical Branch, Galveston, TX

²University of California in San Francisco, San Francisco, CA

³Biomedical Department of Internal Medicine and Medical Specialties, University of Palermo

⁴Pharmacology & Toxicology, University of Texas Medical Branch, Galveston, TX

⁴Euro-Mediterranean Institute of Science and Technology, Palermo, Italy

Abstract

Backgrounds and aims—Low concentrations of plasma HDL-C are associated with the development of atherosclerotic cardiovascular diseases and type 2 diabetes. Here we aimed to explore the relationship between the *in vivo* fractional synthesis of triglycerides (f_{TG}) in subcutaneous (s.q.) abdominal adipose tissue (AT), HDL-C concentrations and HDL particle size composition in non-diabetic humans.

Methods—The f_{TG} in s.q. abdominal AT was measured in 16 non-diabetic volunteers (7 women, 9 men; Age: 49 ± 20 years; BMI: 31 ± 5 kg/m; Fasting Plasma Glucose: 90 ± 10 mg/dl) after 2H_2O labeling. HDL-C concentration and subclasses, large (L-HDL), intermediate (I-HDL) and small (S-HDL) were measured.

Results—Linear regression analyses demonstrated significant associations of f_{TG} with plasma concentration of HDL-C ($r=0.625, p=0.009$) and percent contribution of L-HDL ($r=0.798, p<0.001$), I-HDL ($r=-0.765, p<0.001$) and S-HDL ($r=-0.629, p=0.009$). When analyses

Corresponding Author: Demidmaa Tuvdendorj, MD, PhD, Division of Endocrinology, Department of Internal Medicine, University of Texas Medical Branch, Galveston, TX, Tel: 409 772 1962, 409 772 1922, detuuden@utmb.edu.

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Conflict of interest

The authors declared that they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

Author contributions

Conduction of clinical studies: DT, AOM, MC, NA.

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were performed by gender, the associations remained significant in women (HDL-C: $r=0.822, p=0.023$; L-HDL: $r=0.892, p=0.007$; I-HDL: $r=-0.927, p=0.003$) but not men.

Conclusions—Our study demonstrated an *in vivo* association between subcutaneous abdominal adipose tissue lipid dynamics and HDL parameters in humans, but this was true for women not men. Positive association with L-HDL and negative with I-HDL suggest that subcutaneous abdominal adipose tissue lipid dynamics may play an important role in production of mature functional HDL particles. Further studies evaluating the mechanism responsible for these associations and the observed gender differences are important and warranted to identify potential novel targets of intervention to increase the production of atheroprotective subclasses of HDL-Cs and thus decreasing the risks of development of atherosclerotic conditions.

Keywords

triglycerides; adipocyte; HDL-C metabolism; stable isotope tracer kinetics; atheroprotective; gender-related; mass spectrometry

Introduction

A low concentration of plasma high density lipoprotein cholesterol (HDL-C) is associated with insulin resistance (IR), atherosclerotic cardiovascular diseases (CVDs) and type 2 diabetes mellitus (T2DM) (1-6). A 15-30 % lower concentration of HDL-C has been observed in patients with T2DM and impaired glucose tolerance (IGT) (7,8). Despite this well-established association, recent therapeutic interventions to increase plasma concentration of HDL-C have failed to demonstrate improved outcomes (9-11). This may suggest that an increase in the amount of total HDL-C may not be enough to protect from CVDs; rather, an increase in functional HDL subclasses or change in HDL metabolism may be more important (12-16). HDL particle assembly occurs in liver and intestine; however, rodent and *in vitro* studies have demonstrated that adipose tissue lipid dynamics appear to play an important role in the maturation of HDL particles and production of functional HDL particles (14,15,17). No human studies have been done, however, looking for an *in vivo* association between adipose tissue lipid dynamics and HDL metabolism.

The dysfunction of adipose tissue, e.g., the reduced ability to accumulate excess calorie intake, has been proposed to play a significant role in the development of IR, T2DM and CVD (18,19). Recently, we have shown that the inability of adipose tissue to synthesize triglycerides (TG) may result in increased spill-over of FFAs into circulation and contribute to the development of IR and the metabolic syndrome (20). Thus, in the current study we evaluated the *in vivo* relationship between a parameter of subcutaneous (s.q.) abdominal adipose tissue lipid metabolism and plasma concentrations of HDL-C in non-diabetic adults. As a parameter of s.q. abdominal adipose tissue lipid metabolism we measured the fractional TG synthesis (f_{TG}), which is an estimate of a fraction of adipose tissue TGs that have been newly synthesized over the period of labeling with deuterium water (2H_2O ; 21). Thus we evaluated the associations of f_{TG} with plasma concentration of HDL-C and also the percent distribution of HDL subclasses of different sizes, e.g., large (L-HDLs), intermediate (I-HDLs) and small (S-HDLs). The HDL particles comprise of a highly heterogeneous group of plasma lipoproteins, which differ by their density and the composition of specific proteins

(22). Although the functions of the different HDL subclasses remain largely unknown (23), the L-HDLs are generally known to be atheroprotective (24), while the I-HDLs and S-HDLs are considered to be lacking this property (25,26). Finally, given the well-established gender differences in HDL-C (27,28), we evaluated gender-related associations as well.

Materials and methods

Subjects

Men and women with stable diet and weight during the previous 6 months and stable medications for 90 days were eligible for the study. Exclusion criteria were any evidence of acute illnesses, diabetes mellitus (defined as fasting plasma glucose (FPG) $> 126 \text{ mg}\cdot\text{dl}^{-1}$ or taking medications that lower glucose), pregnancy or lactation, a history of substance abuse and the inability to provide informed consent. Two subjects (1 woman and 1 man) were taking metoprolol, otherwise no other subjects reported taking any medications known to affect glucose or lipid metabolism. All studies and procedures were approved by the Institutional Review Board at the University of Texas Medical Branch (UTMB), Galveston, TX, and all participants provided signed informed consent.

Experimental protocol

Upon enrollment, subjects received 50 ml of 70% $^2\text{H}_2\text{O}$ (Cambridge Isotopes, Andover, MA, USA), three times a day for one week and 50 ml twice daily, for the next 11 weeks (total of 12 weeks of $^2\text{H}_2\text{O}$ labeling), as previously described (20,21). This approach allows measuring the fraction of adipose tissue TGs that were newly synthesized during the labeling period. Quantitatively, it allows measurement of the degree of incorporation of ^2H into C-H bonds of α -glycerol phosphate, which is proportional to two factors: the fraction of labeled hydrogen atoms in tissue water (i.e., the enrichment of $^2\text{H}_2\text{O}$ in whole body water) and the fraction of C-H bonds in glycerol that are incorporated from body water. Thus, if the body water ^2H enrichment and the number of C-H bonds in glycerol that derive from body water are known, the incorporation of ^2H into H atoms in the glycerol moiety of TGs reveals the fraction of newly synthesized TGs. Thus to measure the body water $^2\text{H}_2\text{O}$ enrichment the saliva samples were collected weekly throughout the labeling period. To measure the incorporation of ^2H into C-H bonds of α -glycerol phosphate, adipose tissue biopsy samples were obtained at the end of the labeling period, as described previously (20). Briefly, subcutaneous abdominal periumbilical adipose tissue biopsy samples were obtained under sterile conditions and local anesthesia with 1 % lidocaine. After a small incision, a biopsy needle (Bard Biopsy Systems, Crawley, UK) was used to obtain tissue samples. Additionally, blood samples were collected to measure clinical chemistry parameters and HDL-C values. The subjects also underwent Dual Energy X-ray Absorptiometry (DEXA, GE Lunar iDXA, GE Medical Systems Lunar, Madison, WI, USA) to measure body fat mass.

Plasma lipid analyses

Fasting blood samples obtained at the end of 12 weeks of $^2\text{H}_2\text{O}$ labeling were used for the measurement of plasma VLDL-C, TG, TC, HDL-C and LDL-C; the analyses were done

using a Vitros 5600 analyzer (Ortho Clinical Diagnostic, Rochester, NY, USA) in the Clinical Pathology Laboratory, UTMB.

The HDL subclass analyses were performed in the laboratory of M.R. and G.M. at the University of Palermo, Italy, in a blinded manner. Non-denaturing, linear polyacrylamide gel electrophoresis was used to separate the HDL subclasses and the measurements were done using the LipoPrint^a System (Quantimetrix Corporation, Redondo Beach, CA, USA) as described below. This method has been validated against gradient gel electrophoresis and nuclear magnetic resonance (reviewed in 29).

Twenty five μl of plasma sample was mixed with 300 μl Lipoprint Loading Gel and placed upon the upper part of the 3% polyacrylamide gel. After 30 min of photopolymerisation at room temperature, electrophoresis was performed for 50 min at 3 mA. Each electrophoresis chamber involved two quality controls. The electrophoresed gels were then scanned, using the LipoPrint^a System, to determine the relative area of each lipoprotein subfraction. After scanning, electrophoretic mobility and the area under the curve were calculated qualitatively and quantitatively. HDL subclasses were distributed as ten bands: HDL-1, HDL-2 and HDL-3 were defined as L-HDL; HDL-4, HDL-5, HDL-6 and HDL-7 were defined as I-HDL; HDL-8, HDL-9 and HDL-10 comprised the S-HDL fraction (30,31). The relative area for each subclass or, in other words, its contribution into total lipoprotein fraction was expressed as a percentage (%). This percent contribution of a subclass was multiplied by the total concentration of plasma HDL-C to yield the concentration of each subclass in $\text{mg}\cdot\text{dl}^{-1}$.

Adipose tissue TG analyses

Adipose tissue lipids were extracted with chloroform:methanol (2:1) Folch extraction; TG were isolated *via* thin layer chromatography. FA methyl esters were separated from glycerol. The fraction containing the glycerol was further derivatized to the glycerol triacetate derivative for GC/MS analysis (20,32). TG-glycerol isotopic enrichments of the glycerol-triacetate derivative were determined by GC-MS (5971 and 5973 models, Hewlett-Packard, Palo Alto, CA, USA), using a DB-225 fused silica column, in methane chemical ionization mode monitoring mass-to-charge ratios (m/z) of 159, 160, 161 for M_0 , M_1 , and M_2 as previously described (20,32).

Measurement of $^2\text{H}_2\text{O}$ enrichments in body water

Enrichment of $^2\text{H}_2\text{O}$ in body water was measured in saliva samples, as previously reported (20). The integrated (AUC) exposure to heavy water for each subject was calculated as the area under the water enrichment time course for individual water enrichment measurements using the trapezoid method.

Calculations for TG-glycerol synthesis from $^2\text{H}_2\text{O}$

TG newly synthesized during the $^2\text{H}_2\text{O}$ administration was calculated as previously described using the following equation:

$$f_{\text{TG}} (\%) = EM_1 [\text{TG} - \text{glycerol}] / A^{\infty}_1 [\text{TG} - \text{glycerol}]$$

where f is the fraction (%) of newly synthesized TG molecules present, EM_1 is the excess mass isotopomer abundance for M_1 -glycerol and A_1^∞ is the asymptotic mass isotopomer M_1 abundance of a fully labeled glycerol, and calculated, as described previously (20,32).

Statistical analyses and data presentations

Data are presented as means \pm SD. The differences in parameters between the groups of men and women were evaluated using 2-tail, unequal distribution variance Student t-test. Relationship analyses were performed using a linear regression model. The $p < 0.05$ was considered statistically significant.

Results

Age, BMI and metabolic characteristics of study subjects

Sixteen non-diabetic adults (9 men and 7 women) participated in the study; the characteristics of the subjects are presented in Table 1 for the entire group and for men and women separately. There was no change in body weight during the deuterium labeling period (data not shown). Men were significantly heavier ($p=0.038$) and taller ($p=0.003$) than the women; however there were no differences in either BMI ($p=0.247$) or percent body fat mass ($p=0.060$) though there was a trend toward greater fat mass in the women. One female subject had impaired fasting glucose concentration of $122 \text{ mg}\cdot\text{dl}^{-1}$. Two women were menopausal (73 and 75 years old) with plasma HDL concentrations of 72 and $42 \text{ mg}\cdot\text{dl}^{-1}$ respectively.

The plasma concentrations of glucose, lipids and HDL subclasses, as well as the % contribution and concentrations of HDL subclasses, are also presented in Table 1.

There were no differences in fasting plasma glucose, TG, VLDL, LDL, total or HDL cholesterol. Interestingly, the percent contributions of S-HDL subclasses were significantly higher ($p=0.004$) in men when compared to women, whereas L-HDL was lower ($p=0.004$) and I-HDL was similar ($p=0.094$; Table 1). The absolute concentration of L-HDL was also significantly higher in women than in men ($p=0.013$), however no differences in concentrations of I-HDL and S-HDL between the groups were observed (Table 1).

Adipose TG kinetics

The $^2\text{H}_2\text{O}$ body water enrichment was stable over the course of the 12 week labeling protocol for individual subjects with an average enrichment of $1.6 \pm 0.4\%$. The average fractional synthesis of TG (f_{TG}) was $17 \pm 6\%$ and there was a non-significant trend towards higher synthesis in women as compared to men ($20 \pm 6\%$ vs. $14 \pm 5\%$, $p=0.057$).

Association between the HDL parameters and f_{TG}

First, we conducted association analyses for the whole group, including both men and women. Linear regression analyses showed that s.q. abdominal adipose tissue f_{TG} significantly and positively correlated with the plasma concentration of total HDL ($r=0.627$, $p=0.009$) and L-HDL ($r=0.936$, $p < 0.001$). However, no association was seen with the concentrations of I-HDL ($r=0.362$, $p=0.168$) or S-HDL ($r=0.205$, $p=0.445$). f_{TG} also

correlated positively with the % contribution of the L-HDL subclass ($r=0.797$, $p<0.001$), but negatively with the % contribution of the I-HDL ($r=-0.763$, $p<0.001$) and S-HDL ($r=-0.628$, $p=0.009$) subclasses.

We then conducted association analyses for men and women separately and the results are depicted in Figure 1. In women, f_{TG} significantly and positively correlated with the plasma concentration of total HDL (Fig. 1A; $p=0.016$) and % of L-HDL subclass (Fig. 1B; $p=0.004$), but negatively with % of I-HDL subclass (Fig. 1C; $p=0.002$). No association was observed with % of S-HDL subclass (Fig. 1D; $p=0.146$). Interestingly, no significant correlations between f_{TG} and plasma concentration of HDL or % contribution of HDL subclasses were observed in the men (Fig. 1E-H).

Discussion

Our results, for the first time, demonstrate an *in vivo* association between s.q. abdominal adipose tissue TG synthesis, a marker of adipose tissue lipid dynamics, and HDL-C metabolism in humans. We also report a gender difference in this association, which suggests a possible link between gender diversity in adipose tissue and HDL metabolism.

Given the atheroprotective effect of HDL, a large body of work has been produced regarding possible mechanisms responsible for decreased plasma HDL-C concentrations in conditions associated with high cardiovascular risk, such as obesity. Although incomplete, the current literature supports two important mechanisms involved in determining low HDL-C (33). The first suggests that with increased plasma TG-rich particles there is increased exchange of TGs for cholesterol esters in HDL and LDL particles. Thus HDL particles become highly TG-enriched and are more easily degradable (34,35). This mechanism suggests an indirect association between HDL metabolism and adipose tissue lipid dynamics. When adipose tissue is unable to efficiently store TGs there is increased FFA efflux from adipose tissue (18-20) and, subsequently, an increase in plasma TG-rich particles. The second proposed mechanism suggests that due to decreased efflux of cholesterol from adipose tissue, the lipidation process of HDL particles is inadequate and, thus, the HDL particles are unable to mature resulting in particles which are catabolized at higher rates (14,15,17,36-38). ATP-binding cassette transporter A1 (ABCA1) and Scavenger receptor class B member 1 (SR-B1) have been shown to play a significant role in this process of HDL lipidation (14,15,17). People with Tangier disease have mutation in *ABCA1* and have extremely low levels of HDL-C and apoA-1 due to high rapid catabolism of the apolipoprotein as a result of its being poorly lipidated (39,40). Genetic studies have shown that some rare alleles of *ABCA1* associate with low levels of HDL-C and decreased efflux from monocyte-derived macrophages (41). These data demonstrate the significance of HDL-C lipidation and the role of ABCA1 and SR-B1 in this process. Our results and the published data (14,15,17,36-38) suggest a direct association between HDL metabolism and adipose tissue lipid dynamics. Though our study did not address the potential mechanisms involved, this is the first human *in vivo* support for an association between HDL metabolism and adipose tissue lipid dynamics in non-diabetic humans, independent of triglyceride elevation.

Our study also suggests a gender-specific association between the lipid dynamics in s.q. abdominal adipose tissue and HDL parameters (Fig 1). Women have higher plasma HDL-C concentrations compared to men, and specifically have elevated levels of large apoA-I particles (26-28,42,43). In our study, we did not observe differences in plasma concentration of total HDL-C between men and women, which may be explained by small sample size (Table 1). However, as previously shown (42), we did observe that the percent distribution of L-HDL particles is higher in women when compared to men, whereas that of S-HDL particles is higher in men (Table 1). We also observed that the percent distribution of L-HDLs positively correlated with s.q. abdominal adipose tissue TG synthesis in women (Fig. 1B), but not men (Fig 1F). If adipose tissue lipid dynamics are associated with HDL metabolism (e.g., production of functional HDL particles) this gender specific difference in these associations raises several questions: 1) how does hormonal status affect this interaction; and 2) how do different fat depots affect this interaction (43-45), e.g., abdominal vs. gluteal? Our current study does not answer these questions; however they deserve attention in future studies.

To evaluate the function of adipose tissue, we measured its ability to synthesize TGs. In our previous report on TG synthesis (20) we reported the f_{TG} data from ten subjects presented here and we demonstrated that f_{TG} associated positively with tissue insulin sensitivity, but negatively with plasma levels of FFAs. We hypothesized that the inability of adipose to accumulate excess calorie intake, or the inability of adipocytes to mature, may result in increased efflux of FFAs into circulation, which ultimately may result in ectopic fat deposition and the development of tissue insulin resistance (18-20). Thus f_{TG} may represent a kinetic marker of adipose tissue lipid dynamics. Interestingly, *in vitro* studies demonstrated that stimulation of adipose tissue lipolysis results in increased efflux of both glycerol, a marker of lipolysis, and cholesterol (17,43,44,46,47). Our study subjects maintained stable body weight during the deuterium labeling and in the steady state TG synthesis should equal the TG lipolysis. Thus subjects with higher f_{TG} should have had higher TG lipolysis and higher cholesterol efflux from adipocytes. The impairment in adipocyte lipid dynamics may associate with both impaired TG turnover and cholesterol efflux/metabolism. If this hypothesis is true, it can partially explain low concentration of plasma HDL-C in metabolically compromised subjects, such as the obese and insulin resistant.

There are several limitations of this study. First, we have a relatively small sample size, which may explain the lack of difference in HDL concentrations between men and women. Second, we include pre and post-menopausal women. While it is well established post-menopausal women have on average a lower HDL-C (48), the two postmenopausal women (age: 73 and 75 years old) in our study had plasma HDL-C concentrations of 72 and 42 $\text{mg}\cdot\text{dl}^{-1}$, respectively, which may suggest that, at least in this small study and especially in the group of women, there was no effect of age or hormonal status on the results and conclusions. Third, and perhaps most importantly, this is a cross-sectional study which does not reveal cause and effect relationships but rather is hypothesis generating for future mechanistic investigations.

In conclusion, our study for the first time demonstrated an *in vivo* association between s.q. abdominal adipose tissue lipid dynamics (e.g., f_{TG}) and HDL parameters (e.g., plasma

concentration of total HDL-C and relative contributions of HDL subtypes) in women but not men. Positive association with L-HDL and a negative association with I-HDL suggest that s.q. abdominal adipose tissue lipid dynamics may be important for the production of mature functional HDL particles and may provide new targets for developing therapeutic strategies to decrease cardiovascular risks. Further studies evaluating the mechanism responsible for these associations and the observed gender differences are important and warranted.

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Abbreviations

f_{TG}	fractional synthesis of Triglycerides
L-HDL	Large HDL
I-HDL	Intermediate HDL
S-HDL	Small HDL
EM₁	excess mass isotopomer abundance for M ₁ -glycerol
A[∞]₁	asymptotic mass isotopomer M ₁ abundance of a fully labeled glycerol

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Highlights

- Adipose tissue (AT) lipid dynamics associates with HDL-C metabolism in humans
- However this association is gender-specific
- AT triglyceride synthesis directly associates with large HDL-C particles
- AT triglyceride synthesis inversely associates with intermediate HDL-C particles
- Modulation of AT lipid dynamics may help prevent the development of atherosclerosis

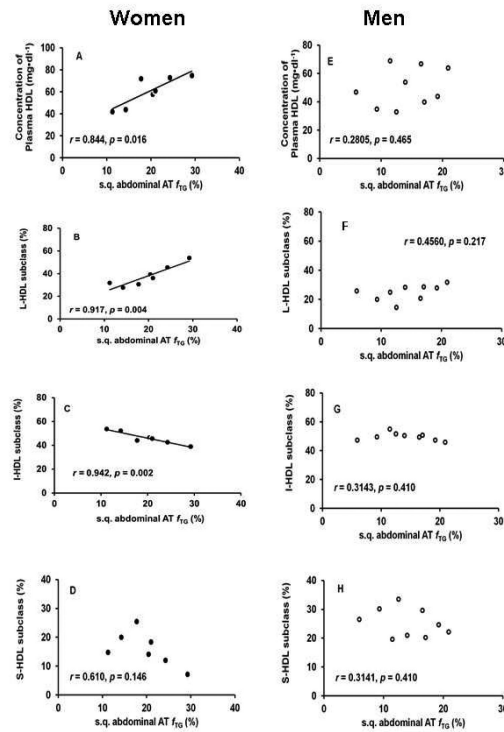


Fig. 1. Association analyses between adipose tissue lipid kinetics and parameters of HDL-C metabolism

Association analyses between fractional triglyceride synthesis (f_{TG}) in s.q. abdominal adipose tissue and plasma concentration of total HDL, the percent contribution of large, intermediate and small HDL (L-HDL, I-HDL and S-HDL, respectively) subclasses in men and women were performed using linear regression model. The $p < 0.05$ was considered statistically significant. (A) f_{TG} significantly and positively associated with the concentrations of plasma total HDL, (B) and percent contribution of L-HDL subclass, and (C) negatively with percent contribution of I-HDL subclass in women, (E, F, G) but not in men. (D, H) No significant correlations were observed between f_{TG} and the percent contribution of S-HDL in either group.

Table 1

Metabolic characteristics of study subjects.

Parameters	All (n=16)	Women (n=7)	Men (n=9)	<i>p</i> value
Age (years)	49±20	48±18	50±22	0.898
Racial distribution				
African American	3	1	2	-
Caucasian	13	6	7	-
Body weight (kg)		77±19	98±17	0.038
Height (cm)		160±6	172±7	0.003
Body mass index (kg•m ⁻²)	31±5	30±6	33±3	0.247
% Body fat mass (%)	39±6	42±7	36±5	0.060
Fasting glucose (mg•dl ⁻¹)	90±10	92±14	88±6	0.440
Plasma triglyceride (mg•dl ⁻¹)	109±70	81±40	131±83	0.171
Plasma VLDL triglyceride (mg•dl ⁻¹)	22±14	16±8	26±17	0.168
Plasma total cholesterol (mg•dl ⁻¹)	180±34	186±32	176±36	0.571
Plasma LDL cholesterol (mg•dl ⁻¹)	104±28	109±25	99±30	0.509
Plasma HDL cholesterol (mg•dl ⁻¹)	55±14	61±14	50±14	0.156
L-HDL subclass (%)	31±10	38±9	25±3	0.004
I-HDL subclass (%)	48±4	46±5	50±3	0.094
S-HDL subclass (%)	21±7	16±6	25±5	0.004
L-HDL subclass (mg•dl ⁻¹)	18±9	24±10	13±5	0.013
I-HDL subclass (mg•dl ⁻¹)	26±6	27±4	25±7	0.448
S-HDL subclass (mg•dl ⁻¹)	11±4	10±4	12±3	0.152

Data are presented as Mean±SD. The differences between the groups were evaluated using 2-tail, unequal distribution variance Student t-test. *p*<0.05 was considered statistically significant.