

Oxidative and inflammatory status and HDL functions of obese pre and post menopausal women

[Obez pre ve postmenopazal kadınların oksidatif ve inflamatuvar durumları ve HDL fonksiyonları]

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Registered: 20 December 2012; Accepted: 16 December 2013]

[Kayıt Tarihi: 20 Aralık 2012; Kabul Tarihi: 16 Aralık 2013]

ABSTRACT

Objective: Obesity and age related vascular changes coupled with the effect of estrogen withdrawal increases predisposition to atherosclerosis in postmenopausal women. But the functions of high density lipoprotein (HDL) were not well established in postmenopausal women. In the present study, we mainly aimed to evaluate the changes in the functions of HDL and aimed to measure, lipid peroxidation, lipid profile and homocysteine levels as a supporting evidence in pre and postmenopausal obese women as a result of estrogen depletion.

Material and Methods: This study included 20 premenopausal, 22 postmenopausal 42 obese (BMI>30 kg/m²) and 26 premenopausal non-obese. These twenty six premenopausal women with normal BMI (20<BMI<25 kg/m²) were recruited to serve as the control group. Markers of cardiovascular disease (CVD), namely high sensitive C-reactive protein (hsCRP), homocysteine and lipoprotein(a) (Lp(a)) were measured. Glucose, lipid parameters and Malondialdehyde (MDA), antiinflammatory HDL levels were also measured. Functional properties of HDL were determined by the change in fluorescence intensity resulting from oxidation of DCFH (Dichloroflorescein) by LDL in the presence of patient HDL in cell free serum.

Results: Lipid profiles were impaired in both pre and postmenopausal obese women. CVD markers and glucose levels increased in post menopausal women. But hsCRP levels were also increased in premenopausal compared to control group. MDA, the product of lipid peroxidation was increased in both pre and postmenopausal women. Antiinflammatory characteristics of HDL were only impaired in postmenopausal women.

Conclusion: Postmenopausal obese women have lost their antiinflammatory HDL functions. This situation can be a significant indicator of endothelial dysfunction and cardiovascular disease in postmenopausal women. But the increase in oxidative stress was observed in all of the obese patients regardless of estrogen withdrawal.

Key Words: Menopause, HDL functions, homocystein, malondialdehyde

Conflict of Interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

ÖZET

Amaç: Obezite ve östrojen yokluğunun etkisiyle eşlik eden vasküler değişiklikler menopoz sonrası kadınlarda ateroskleroza yatkınlığı artırmaktadır. Ancak, yüksek yoğunluklu lipoprotein (HDL) fonksiyonları postmenopozal kadınlarda detaylı bir şekilde incelenmemiştir. Bu çalışmada, premenopozal ve östrojen azalmasının sonucu olarak postmenopozal obez kadınlarda esas olarak HDL'nin fonksiyonlarını ve destekleyici bilgi olarak lipit peroksidasyonu, lipit profili ve homosistein düzeylerindeki değişiklikleri değerlendirmeyi amaçladık.

Gereç ve Yöntemler: Bu çalışma, 20 premenopozal, 22'si postmenopozal 42 obez (VKI> 30 kg/m²) ve 26 premenopozal non-obez örneği içermektedir. Bu 26 normal VKI'lı (20<VKI<25 kg/m²) kadın kontrol grubu olarak değerlendirilmiştir. Kardiyovasküler hastalık (CVD) belirteçleri olarak, yüksek hassasiyetli C-reaktif protein (hsCRP), homosistein ve lipoprotein (a) (Lp(a)) ölçülmüştür. Ayrıca Glukoz, lipit parametreleri ve malondialdehit (MDA), antiinflatuar HDL düzeyleri de değerlendirilmiştir. HDL'nin fonksiyonel özellikleri, hücresiz serumda hasta HDL'sinin varlığında DCFH'nin (Dikloroflorescein) LDL tarafından oksidasyonu ile oluşan floresan şiddetindeki değişim ile belirlenmiştir.

Bulgular: Lipit profilleri, pre ve postmenopozal kadınlarda bozulmuştur. CVD belirteçleri ve glukoz düzeyleri sadece postmenopozal kadınlarda artarken, hsCRP düzeyleri premenopozal kadınlarda kontrol grubuna göre artmıştır. Lipit peroksidasyonunun bir ürünü olan MDA, hem pre hem de postmenopozal kadınlarda artmıştır. Antiinflatuar HDL özellikleri ise sadece postmenopozal kadınlarda bozulmuştur.

Sonuç: Postmenopozal obez kadınlar antiinflatuar HDL fonksiyonlarını kaybetmiştir. Bu durum, endotel disfonksiyonunun ve kardiyovasküler hastalıkların önemli bir belirtici olabilir. Fakat oksidatif stresteki artışlar östrojen azalmasına bakılmaksızın obez hastaların tamamında gözlenmiştir.

Anahtar Kelimeler: Menopoz, HDL fonksiyonları, homosistein, malondialdehit

Çıkar Çatışması: Yazarlar çıkar çatışması beyan etmemiştir.

Introduction

Cardiovascular Disease (CVD) is the most important cause of death for post menopausal women [1]. Changes in estrogen levels and activity cause an increase in predisposition to CVD [2,3]. It has become clear that estrogen effects lipoprotein metabolism and profile at many points and in many potentially beneficial ways where estrogen as a prophylactic for cardiovascular disease prevention. Estrogen causes higher HDL cholesterol levels and lower LDL cholesterol levels, reduces total cholesterol and LDL cholesterol and plasma levels of lipoprotein (a) (Lp[a]). Also, HDL increases apo B gene transcription and enhances LDL catabolism, presumably due to increased expression of LDL receptors. The rise in HDL and apo A-I is primarily attributable to increased synthesis with decreased clearance of HDL particles through diminished hepatic lipase activity playing a lesser role [4].

Weight gain is accelerated at menopause. Especially depletion in estrogen causes an increase in abdominal fat. Recent studies reported that adipose tissue is metabolically active and promotes inflammation [5]. These changes in the body status are associated with an increase in biomarkers of endothelial inflammation and vascular oxidative stress [6]. Both obesity and postmenopausal status are high cardiovascular risk factors. Previous studies have indicated that improving endothelial functions, inflammation and oxidative status may prevent atherosclerosis [7].

Functions of high density lipoprotein (HDL) are as important as its quantity [8]. HDL removes excess cholesterol from peripheral tissues by the reverse cholesterol transport pathway and inhibits LDL (Low density lipoprotein) oxidation [9]. These beneficial effects of HDL against LDL oxidation have been attributed to different proteins such as lecithin-cholesterol acyltransferase (LCAT) and platelet-activating factor acetyl hydrolase (PAF-AH) and paraoxonase-1 (PON-1) [10]. PON-1, which catalyzes the hydrolysis of biologically active lipids in oxidized LDL is one such protein that has been reported to possess both anti-oxidant and anti-inflammatory properties [9-11]. In this study, we aimed to determine the functionality of HDL, lipid profile and peroxidation as well as homocysteine levels in pre and post menopausal obese women in order to evaluate the role of estrogen depletion on cardiovascular risk factors.

Materials and Methods

Subjects

Forty two obese women were included in this study. Women with body mass index (BMI) greater than 30 kg/m² were considered obese. Of these patients 22 were postmenopausal, 20 were premenopausal. Women were considered postmenopausal if their menses had ceased naturally for at least 12 months. Also 26 premenopausal

non-obese woman with normal BMI (20<BMI<25 kg/m²) were recruited to serve as the control group. Patients with any metabolic disorder, such as diabetes mellitus, hypo or hyperthyroidism or renal dysfunction, who were smokers, using any cholesterol-lowering drugs and alcohol were excluded. None of the patients exercise regularly. This exclusion criteria was also valid for control subjects. Also all patients included in these study had normal HDL-C levels (≥ 50 mg/dl).

The study protocol was approved by Ethical Committee of Eskişehir Osmangazi University and informed consent was received from all participants (30.06.09/41).

Methods

Biochemical analysis:

Fasting blood samples of subjects were collected to obtain serum with BD Vacutainer gel separator tubes. Total cholesterol (TC), LDL-cholesterol (LDL-C), HDL-cholesterol (HDL-C), triglyceride (TG) and glucose levels were measured using enzymatic colorimetric assays and apolipoprotein A-1 (Apo A-1), Apolipoprotein B (Apo B), Lipoprotein (a) (Lp (a)), high sensitive C reactive protein (hsCRP) levels were determined immediately by immunoturbidimetric methods using Modular Systems (Roche Diagnostics, Switzerland) according to manufacturers instructions and 1 ml of serum samples were stored at -80 °C within one hour of collection until the time of measuring MDA, Homocystein levels and HDL functions. Serum malondialdehyde (MDA) levels were measured according to the method described by Ohkawa et al [12]. The results were expressed as nmol ml⁻¹. Serum homocysteine levels were measured by ELISA with Diazyme kits (San Diego, CA, USA) according to manufacturers instructions.

The antiinflammatory capacity of HDL

Functional properties of HDL were determined by the change in fluorescence intensity resulting from oxidation of DCFH (Dichloroflorescein) by LDL in the presence of patient HDL [13]. Previously a cell-free assay that measures functional properties of HDL by testing the effect of HDL on the production of reactive oxygen species after oxidation and conversion of dichlorodihydrofluorescein diacetate (DCF-DA) to fluorescent DCF (2',7'-dichlorofluorescein) was developed to provide an alternative to cell-based assays [14]

HDL content of patient sample was adjusted to 10 µg/ml cholesterol after the isolation of HDL by dextran sulfate [15]. For precipitation with dextran sulfate, Sigma HDL cholesterol reagent containing dextran sulfate and magnesium ions was dissolved in distilled water.

DCFH-DA was dissolved in fresh methanol at 2.0 mg/ml and was incubated at room temperature and protected from light for 30 min. This results in the release of DCFH. On interaction with lipid oxidation products DCFH forms DCF, which produces intense fluorescence

(13). Fifty microliters of dextran sulfate (1.0 mg/ml) was mixed with 500 µl of the test serum and incubated at room temperature for 5 min and subsequently centrifuged at 3,000 g for 10 min. The supernatant containing HDL was used in the experiments. All supernatants which were obtained from every patient serum had same amount of HDL. 200 µl of the isolated LDL solution from a serum pool (adjusted to 50 µg/ml) and 900 µl of test HDL (adjusted to 10 µg/ml cholesterol) were incubated in a tube at 20-25 °C and in a dark room for 1 hour. 100 µl of DCFH solution (0.2 mg/ml) was then added to each well and incubated for 2 hours more. Fluorescence intensity was determined with spectrofluorometer (Jasco FP-750) set at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Values of fluorescence units (FU) (HDL Score) were normalized to 1.0 as the positive control (13). Fluorescence unite (FU) (HDL skor value) which is obtained by oxidation of LDL with DCFH on the absence of HDL was accepted 1. FU was determined by change in the fluorescence intensity which occurs after oxidation of DCFH by LDL in the

presence of HDL. Every unit of Fluorescence intensity which caused by DCF was evaluated as 1 Unit (FU). Values >1.0 FU after the addition of test HDL indicated as proinflammatory HDL; values <1.0 indicated as anti-inflammatory HDL (16). Slit width was 6 mm. Intra-assay and inter-assay precision of study was 3% and 5% respectively.

Statistical Analysis:

Statistical Package for the Social Science (SPSS) for Windows 13.0 was used for statistical analysis. Data was expressed as Mean ± SD. Comparisons were performed by one way ANOVA (tukey for post hoc analyses) test for normally distributed continuous variables while Kruskal-Wallis H test for non-normally distributed variables for several independent samples.

Results

Characteristics and studied biochemical parameters of the patients and the control group are shown in Table 1. Postmenopausal women were slightly elder than both obese

Table 1. Comparison of characteristics and biochemical parameters of control, obese pre- and post menopausal women

Parameters	Premenopause		Premenopause
	non-Obese (control) (n=26)	Obese (n=20)	Obese (n=22)
Age (years)	44,7±11,7	40,2±8,9	56,3±8,4 ^{c,e}
BMI (kg/m ²)	23,9±3,0	36,3±8,3 ^c	35,1±4,3 ^c
Glucose (mg/dl) (mmol/L)	70,8±11,7 1276,5±210,9	77,7±14,5 1333,3±398,2	90,0±16,2 ^{c,d} 1606,3±301,7 ^{c,d}
Triglyceride (mg/dl)	105,8±31,5	107,9±40,4	128,7±62,9
Total cholesterol (mg/dl)	190, 0±25,9	210,0±23,7 ^a	211,7±27,7 ^a
HDL-C (mg/dl)	60,1±9,6	55,7±9,8	57,0±7,4
LDL-C (mg/dl)	100,8±22,6	121,8±24,4 ^a	125,7±20,5 ^a
Lp (a) (mg/dl)	30,4±13,0	46,5±31,9	49,0±32,5 ^a
Apo A (mg/dl)	172,3±17,1	167,9±15,5	164,7±22,8
Apo B (mg/dl)	79,5±19,3	82,9±16,7	88,9±15,4
hsCRP (mg/dl)	1,9±1,7	6,8±4,9 ^b	5,5±4,3 ^b
Homocysteine (µmol/l))	8,4±1,1	8,7±1,6	11,9±3,7 ^{a,e}
MDA (nmol/ml)	8,5±1,6	14,8±3,6 ^c	16,7±2,4 ^c

a*: significant differences from non-obese premenopause women (controls) (p<0.05) b**: significant differences from controls (p<0.01). c***: significant differences from controls (p<0.001). d*: significant differences from obese pre menopausal women (p<0.05). e***: significant differences from obese pre menopausal women (p<0.001).

premenopausal women and the non-obese premenopausal women (controls). Both pre and postmenopausal women body mass indices (BMIs) were statistically increased compared to controls indicating an obese state of them ($p < 0.001$ for both). Postmenopausal obese women fasting glucose levels were higher compared to controls ($p < 0.001$) and premenopausal obese women ($p < 0.05$). Also total cholesterol levels were higher in both pre and postmenopausal obese women compared to controls ($p < 0.05$ for both). On the other hand, there were no significant differences between HDL-C levels of the groups. Post and premenopausal obese women LDL-C levels were significantly higher compared to controls ($p < 0.05$). But Lp (a) levels were only high in postmenopausal obese women compared to controls ($p < 0.05$). hsCRP levels were higher in pre and post menopausal obese women compared to controls ($p < 0.01$ for both). Also, postmenopausal obese women homocysteine levels were significantly higher than both controls ($p < 0.05$) and premenopausal obese women ($p < 0.001$) indicating withdrawal of estrogen may increase cardiovascular disease risk. Serum MDA levels of both pre and postmenopausal obese women were increased compared to controls ($p < 0.001$).

Antiinflammatory characteristics of HDL in control, pre

and postmenopausal obese women are given in Figure 1. According to our results, post-menopausal obese women had more proinflammatory HDL [72.7 % (95 percent confidence interval, 1.036-1.164) (median HDL score value 1.04) when compared with premenopausal obese women and controls [25 % (95 percent confidence interval, 0.9175-1.042) (median HDL score value 0.97) and 7.7 % (95 percent confidence interval, 0.847-0.975) (median HDL score value 0.85), $p < 0.01$ and $p < 0.001$, respectively]. The difference between premenopausal obese women and controls was not statistically significant. The median HDL score value of controls (0.85) was similar to median HDL score value of controls (0.87) which was given in our previous study. (16).

Discussion:

Obesity and age related vascular changes coupled with the effect of estrogen withdrawal predispose to changes in endothelial biology in postmenopausal women. Decline in estrogen secretion is associated with an increase in abdominal fat and metabolic diseases which are associated with an increase in CVD risk [15]. Also, decrease in HDL levels and impaired lipid profiles are risk factor for endothelial dysfunction and CVD [17]. In the present

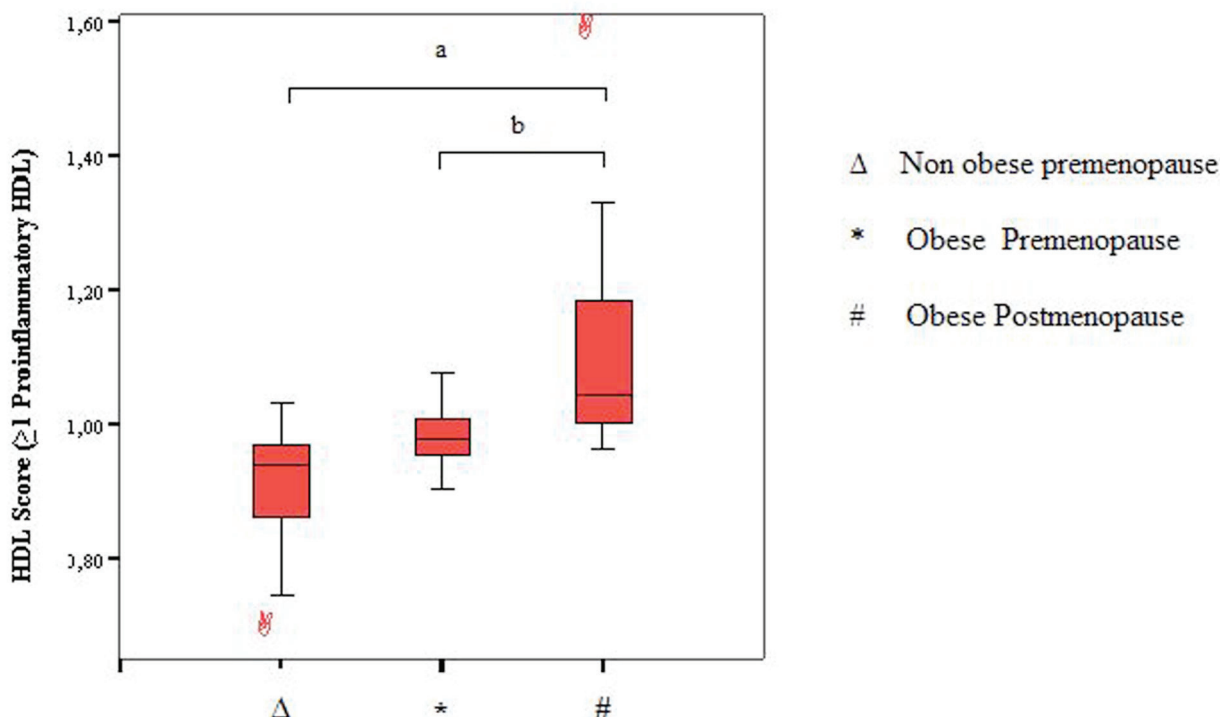


Figure 1. Inflammatory characteristics of HDL in control, pre and postmenopausal obese women.

Figure Footnotes:

Post-menopausal obese women had more proinflammatory HDL [72.7 % (95 percent confidence interval, 1.036-1.164)] when compared with premenopausal obese women and non-obese premenopausal women (controls) [25 % (95 percent confidence interval, 0.9175-1.042) and 7.7 % (95 percent confidence interval, 0.847-0.975)],

a: significantly different from non-obese premenopausal women (controls) ($p < 0.001$)

b: significantly different from premenopausal obese women ($p < 0.01$).

study, we mainly aimed to evaluate the changes in the functions of HDL and aimed to measure, lipid peroxidation, lipid profile and homocysteine levels as a supporting evidence in pre and postmenopausal obese women as a result of estrogen. In our study post menopausal and premenopausal women body mass indices were fairly increased indicating an obese state of these women. Also glucose levels were increased in postmenopausal women. Estrogen withdrawal increased glucose levels which indicate a predisposition to Type II Diabetes Mellitus. In obese non-diabetic individuals oxidative stress is viewed as a likely causative factor in the dysregulation of glucose metabolism and the development of insulin resistance [18]. Although we did not measure insulin resistance in these subjects, the increases in glucose levels may show impaired glucose metabolism in these patients.

It is known that in post menopausal women lipid and lipoprotein metabolism is markedly altered [19]. In the present study, we studied lipid profiles of both pre and postmenopausal obese women. Total cholesterol and LDL-C levels were similarly increased in both pre and postmenopausal obese women compared to controls. Contrary to some other studies, there were no significant differences between lipid profiles of pre and postmenopausal obese women [20-21]. Judith et al. showed marked endothelial dysfunction with an increase in LDL cholesterol and a decrease in flow-mediated-dilatation (FMD) within months of estrogen withdrawal [2]. The results of our study have shown that obesity is a primary factor for the changes of lipid profiles.

On the other hand, adipose tissue is associated with an increase in biomarkers of endothelial inflammation and vascular oxidative stress [6]. Abdominal adipose tissue deposition observed after menopause represents an important source of cytokine production, which would in turn stimulates hepatic CRP production [22-23]. In line with our study Kassi et al showed a positive correlation between BMI and hsCRP levels indicating an inflammatory status in obesity [19]. In another study it was shown that healthy postmenopausal women showed moderate increases in CRP levels within the range of 1.5-7.3 mg/L and had a graded 2-5 fold increase in the risk of future cardiovascular events [24]. In our study, hsCRP levels of pre and postmenopausal obese women were not significantly different. Thus increases in hsCRP levels were not under the effect of estrogen withdrawal and seems to be linked more to obesity and endothelial dysfunction, favoring the atherosclerotic process and cardiovascular events [25]. In our study, homocysteine levels were significantly increased in post menopausal obese women but did not change in premenopausal obese women when compared with controls. This increase shows effects of estrogen withdrawal. A 5- μ mol/L tHcy increment elevates CAD risk by as much as cholesterol increases of 0.5 mmol/L (20 mg/dL) as indicated in a study which is carried out by Boushey et al (26). Es-

trogen promotes vasodilatation through stimulation of endothelial nitric oxide synthases (eNOS) and increase in nitric oxide (NO) bioavailability [21]. By interfering with the transsulfuration pathway, diminishing homocysteine and preventing its accumulation, estrogen may enhance the net production of glutathione (GSH) and stabilizes NO, which contributes to its beneficial effects on the vasculature [27]. These mechanisms may explain unchanged levels of homocysteine compared to controls in pre-menopausal obese women. Also PON1 which is an antioxidant enzyme associated with HDL has homocysteine thiolactonase activity. Hence, PON1 prevents protein homocysteinylation, which is a process involved in atherogenesis. Homocysteine thiolactonase activity of PON1 could be responsible for its anti-atherosclerotic and anti-oxidant activity in decreasing effects of homocysteine [28]. Also in our study Lp(a) levels were only increased in postmenopausal obese women although their HDL levels were in normal range (HDL-C \geq 50 mg/dl) in line with some other studies [29-31]. The increase of Lp(a) levels after menopause may reflect that Lp (a) levels are sensitive to sex steroid hormones [31] and make postmenopausal obese women more prone to atherosclerosis than premenopausal obese women.

Oxidative stress is known to contribute to atherogenesis process in postmenopausal obese women. Studies have supported a link between body fat mass and oxidative stress via obesity-related elevation of fatty acids [1].

Also obesity related elevation of fatty acids could cause oxidative stress due to the increased mitochondrial uncoupling of β -oxidation [1]. Measurement of the breakdown products such as MDA is the most common approach to determine the degree of lipid peroxidation induced by reactive oxygen species (ROS) [32]. In our study MDA levels of both pre and postmenopausal obese women were significantly increased compared to controls indicating a ROS overproduction in pre and post menopausal obese women. The increase in MDA levels was not associated with estrogen withdrawal.

The decreased activity of PON-1 can depress the ability of circulating HDL particle to protect LDL from oxidation, to participate in reverse cholesterol transport pathway, and to inhibit monocyte-endothelial cell interaction. All these appear to be important in the inflammatory response in artery that promotes atherogenesis. This decreased PON1 activity and the tyrosine residues in Apo A1 may be changed by myeloperoxidase which is a determinant of inflammatory state. Myeloperoxidase uses hydrogen peroxide (H_2O_2) and NO_2^- to generate reactive nitrogen species that nitrate tyrosine residues in animal models of inflammation [33]. Thus, Apo A1 levels and functions may decrease as a result of unfunctional proinflammatory HDL against LDL oxidation and inability of HDL to promote cholesterol efflux by the ATP-binding cassette transporter A-1 (ABCA1) pathway [33] In our study Apo A1 levels did not differ between the groups. Thus this proinflammatory HDL-C levels may be at-

tributed to other enzymes and proteins in HDL such as LCAT, ABCA1 and PAF-AH [8-10]. And these enzymes may be directly influenced by estrogens [34]. In this regard function of HDL is more important than its quantity for preventing LDL oxidation. Although the size of study population was a limitation for us, post menopausal obese women had decreased antiinflammatory HDL levels while pre menopausal obese women did not.

In conclusion, decreases in antiinflammatory HDL levels which show rate distribution between HDL fractions and impaired lipid profiles can be a significant indicator of endothelial dysfunction and cardiovascular disease in postmenopausal obese women. Post menopausal obese women lost their anti-inflammatory functions of HDL and normal endothelial functions. These changes might be attributed to decreased estrogen levels in this period which in turn increase the cardiovascular event risk in this women. But increased MDA, and hsCRP levels in these patients possibly result from obesity and these are independent from estrogen withdrawal.

Acknowledgement: We thank to research asistant MSc. Ahmet Musmul for statistical evaluation of the study data.

Conflict of Interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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