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# Circulating small dense LDL, endothelial injuring factors and fibronectin in healthy postmenopausal women

M.L. Muzzio [a,1], G. Berg [a,1], V. Zago [a], F. Basilio [b], S. Sanguinetti [a], G. Lopez [a], F. Brites [a], R. Wikinski [a], L. Schreier [a,*]

[a] *Laboratory of Lipids and Lipoproteins, Department of Clinical Biochemistry, Faculty of Pharmacy and Biochemistry, University of Buenos Aires, Argentina*
[b] *Gynecology Division, Durand Hospital, Buenos Aires, Argentina*

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**Abstract**

*Background:* In postmenopausal women (PMW), an adverse lipoprotein pattern and high risk of coronary artery disease has been described. Studies of the mechanisms promoting the higher atherogenic risk observed in healthy PMW are relevant. We evaluated the interactions among several circulating factors involved in the endothelial injury and inflammation in relation to LDL characteristics, beyond LDL cholesterol.
*Methods:* Lipoprotein profile, including apolipoproteins A-I and B, small dense LDL, hepatic lipase, cholesterol transfer protein (CETP), LDL composition and oxidability were assessed in PMW ($n=30$) in comparison to premenopausal (PreMW, $n=28$). The following emerging factors were measured: homocysteine, phospholipase A2, ferritin, hs-CRP and fibronectin from extracellular vascular matrix. Insulin-resistance was evaluated by waist circumference, HOMA and TG/HDL cholesterol ratios.
*Results:* The risk index apo B/apo A-I was significantly increased in PMW ($p<0.0001$), PMW showed higher proportion of small dense LDL which correlated with the increase in hepatic lipase activity ($p<0.005$) and with insulin-resistance markers ($p<0.05$), but not with CETP. Phospholipase A2 ($p<0.05$), homocysteine ($p<0.005$), hs-CRP ($p<0.005$), fibronectin ($p<0.05$) and ferritin ($p<0.0001$) were increased in PMW. LDL oxidability positively correlated with waist ($p<0.02$), homocysteine ($p<0.05$), fibronectin ($p<0.05$), hs-CRP ($p<0.04$), phospholipase A2 ($p<0.05$), and small dense LDL ($p<0.01$). After adjusting by menopausal condition, age and waist, LDL oxidability remained associated with waist ($\beta$: 0.35, $p=0.047$), homocysteine ($\beta$: 0,36 $p<0,038$), fibronectin ($\beta$: 0,41 $p=0.05$), and small dense LDL ($\beta$: 0.36, $p=0.027$).
*Conclusions:* Evaluation of classic and non-traditional circulating risk factors in hypoestrogenism reflected endothelial and subendothelial inflammation and subclinical atherogenic processes.


*Keywords:* Menopause; LDL oxidability; Fibronectin; A2; Homocysteine; Hepatic lipase

## 1. Introduction

It is widely accepted that the risk of coronary artery disease progressively increases in women after menopause, mainly associated with a reduction in circulating estrogens and an elevation in the plasma levels of LDL [1,2]. In postmenopausal women (PMW), an adverse lipoprotein pattern has been described, even though HDL cholesterol levels tend to be maintained or slightly decreased [3].

Hepatic lipase is a key enzyme involved in the hydrolysis of triglycerides (TG) and phospholipids contained in LDL and HDL particles, and, in cooperation with cholesteryl ester transfer protein (CETP), it also contributes to lipoprotein remodeling. We previously reported a high hepatic lipase activity in menopause [4], likely associated with the fall of endogenous estrogens. A consequence of higher hepatic lipase activity may be the increased of small dense LDL subfraction, already described in PMW [5,6]. This particle *per se* has a greater atherogenic potential than other LDL subclasses [7].

Since estrogens are considered potent antioxidants, an increase in reactive oxygen species and lipid peroxidation would be expected after menopause. The increase in oxidative stress may influence endothelial injury. LDL oxidation

* Corresponding author. Junin 956 (1113) Buenos Aires, Argentina. Tel.: +54 11 4964 8297.
*E-mail address:* lipids@ffyb.uba.ar (L. Schreier).
[1] Contributed equally to this work.

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depends on several factors arising from plasma, as well as from the lipoprotein particle itself. The assessment of novel factors related to LDL oxidation, which could be altered in the postmenopausal condition even in apparently healthy women, would contribute to explain the subclinical atherogenic process.

Plasma homocysteine, considered a cardiovascular risk factor, is able to increase LDL auto-oxidation [8]. However, results from studies focused on the effect of menopause on plasma homocysteine are controversial [9]. Another factor is the lipoprotein-associated phospholipase A2 (LpPLA2) whose pro- or antiatherogenic role has not been totally elucidated yet, especially in postmenopause [10]. Extracellular matrix components, such as fibronectin, may be released to the circulation in case of endothelial injury and thus, it could act as a marker of the atherogenic phenomenon [11]. It is well known that a concomitant inflammatory process takes place and so, hs-CRP could be also higher [12]. After menstrual cessation, ferritin increases in postmenopause and it could promote LDL oxidation by means of its iron content [13].

Investigations of the mechanisms promoting the higher atherogenic risk observed in healthy PMW are considered still relevant, since controlled randomized clinical trials have failed to demonstrate a decrease in the incidence of cardiovascular events after estrogen administration [14]. Our aim was to evaluate the interactions among several circulating factors involved in the endothelial injury in relation to LDL characteristics, beyond LDL cholesterol concentration.

## 2. Methods

### 2.1. Subjects

A total of 58 healthy women were studied. Thirty of them were PMW, clinically evaluated and consecutively recruited at the Climacteric Section of the Gynecology Division at Durand Hospital, Buenos Aires. Their ages ranged from 43 to 60 y (mean 53 y), with at least 1 y of natural menopause and <10 y of amenorrhea. In all the cases, serum levels of follicle-stimulating hormone (FSH) >40 IU/l confirmed the postmenopausal status. The control group included 28 women in reproductive age (23–44 y, mean 33 y) with normal physical examination and laboratory tests. They were consecutively recruited from patients who were attended at the same Center for their routine health check.

None of the women was included when presenting history or underlying symptoms of diabetes or cardiovascular disease, as well as if they were receiving hormonal, hypolipidemic or any other drug known to modify lipid metabolism. Women were also excluded when receiving antioxidant supplementation or having history of hypothyroidism, neoplasia or renal disorder. Women whose weight had varied more than 5% in the last 6 months were also excluded. Four PMW and 5 premenopausal women (PreMW) consumed 2 to 6 cigarettes/day, while the remaining subjects had been non-smokers for the last 10 y. In no case did alcohol consumption surpassed 10 g/day. PMW and PreMW were not under regular training. Both groups of women had similar diets with the following distribution: 20% proteins,<30% fat and at ≥50% carbohydrates. Calories intake varied according to individual body weight. The weight and height of each patient were measured and BMI calculated as weight (kg)/height ($m^2$) to evaluate the obesity degree. Waist circumference was measured midway between the lateral lower rib margin and the superior anterior iliac crest on a standing position and always by the same investigator. Written informed consent was obtained from each subject before admission to the study, which was approved by the Ethic Committee of the School of Pharmacy and Biochemistry, University of Buenos Aires.

### 2.2. Samples

After a 12-h overnight fast blood samples were drawn. In the PreMW group it was carried out at the follicular phase of the menstrual cycle (day 3–7). Serum or plasma was kept at 4 °C for evaluation of fasting glucose, lipids and lipoproteins (within 48 h) or stored at −70 °C for further determination of insulin, hs-CRP, homocystein, Lp-PLA2, CETP and ferritin and fibronectin. Heparin (60 UI/kg body weight) was administered intravenously for the determination of hepatic lipase activity. Ten minutes later, blood obtained by venipuncture of the contralateral arm was collected in tubes placed in ice, and postheparin plasma was kept at −70 °C until its processing within 30 days.

### 2.3. Analytical procedures

Cholesterol, TG and fasting glucose were measured in serum using commercial enzymatic kits (Roche Diagnostics, Mannheim, Germany) in a Hitachi 727 autoanalyser. HDL and LDL cholesterol were determined by standardized selective precipitation methods. Serum lipid measurements were under good quality control (CV%, routinely <3%). Serum hs-CRP, apo A-I and apo B were determined by immunoturbidimetry (Roche Diagnostics, Mannheim, Germany). Insulin, homocysteine and ferritin were evaluated with IMx analyzer (Abbott Laboratories). In order to estimate insulin-resistance, the following indexes were calculated: TG/HDL cholesterol and HOMA (homeostasis model assessment): fasting insulin (μmol/ml) × fasting glucose (mmol/l)/22.5.

### 2.4. CETP activity

CETP activity was determined in serum samples as described previously [15]. Briefly, the ability of serum to promote the transfer of tritiated cholesteryl esters from a tracer amount of biosynthetically labeled $HDL_3$ ($^3H$–CE–$HDL_3$) (NEN Life Science Products, Boston MA) towards serum apo B-containing lipoproteins was evaluated. Samples were incubated with $^3H$–CE–$HDL_3$ (50 μmol/l cholesterol) and 1.5 mmol/l iodoacetate at 37 °C for 3 h. After incubations, lipoproteins were separated by ultracentrifugation (density=1.070 g/ml) at 250,000 ×*g* and 4 °C for 18 h. Radioactivity was measured both in the supernatant, containing the VLDL–IDL–LDL fraction, as well as in the subnatant, containing the HDL fraction using a Liquid Scintillation Analyzer (Packard 210TR; Packard Instruments, Meridian, CT). Results were expressed as percentage of $^3H$-cholesteryl esters transferred from $HDL_3$ to apo B-containing lipoproteins, per ml, per h. Measurements were all carried out in duplicate within the same assay. The intra-assay CV was 4.9%.

### 2.5. Fibronectin levels

Fibronectin was determined by a developed solid-phase enzyme immunoassay (EIA). Microtiter plates (Polysorp, Invitrogen Life Technologies, Carlsbad, CA) were coated with 100 μl of IgM monoclonal murine antibody diluted 1/100 (SC 18826, Santa Cruz Biotechnology Inc, Santa Cruz CA) raised against fibronectin Cs-1 peptide of human origin, in phosphate-buffered saline (PBS, pH=7.4) during 2 h at 37 °C and then blocked with 1.5% W/V bovine serum albumin (BSA) in PBS. Wells were washed with PBS and both 1/10 PBS diluted samples and fibronectin standard (F-2006, Sigma Aldrich) were applied. Incubation was carried out overnight at 37 °C. A rabbit polyclonal antibody against human fibronectin (100 μl diluted 1/1500) (F-3648, Sigma Aldrich, St. Louis, MO) was used as second antibody. Ligand capture was detected by addition of peroxidase-conjugated antirabbit IgG (1/1500) (A-0545, Sigma Aldrich). Binding of the antibody was detected by adding 2% V/V *O*-phenylenediamine in 30% citrate-phosphate buffer (pH=5) with $H_2O_2$. The reaction was stopped by adding 3 mol/l sulfuric acid. Absorbance at 492 nm was registered employing a microplate reader. The intra-assay CV was 7.5% and the inter-assay CV was 8.1%.

### 2.6. LpPLA2 activity

LpPLA2 activity was measured following the radiometric assay described by Blank et al. [16] with few modifications. Briefly, the separation of the released radiolabeled acetate from the lipid substrate was carried out by phase–phase partitioning and measurement of the radioactivity in the aqueous phase.

Each incubation mixture contained diluted serum and 10 μmol/l 1-hexadecyl-2-[$^3$H]acetyl-glicero-3-phosphocholine (specific activity=25 μCi μmol/l) in PBS buffer (pH=7.4). The tritiated substrate (13.5 Ci mmol/1) was from New England Nucleotides, and the non-tritiated one was from Cayman Chemical (Ann Arbor, MI). Once the substrates were mixed, the solvents were evaporated under a stream of nitrogen and redissolved in PBS. The radioactivity of each sample and sample-blanks was measured using a scintillation counter (Packard 210TR; Packard Instruments, Meridian, CT). Radioactivity of the substrate-buffer was also measured. Results were expressed as μmol/ml h. Measurements were all carried out within the same assay. The intra-assay CV was 5.1%.

### 2.7. Hepatic lipase activity

Hepatic lipase activity in postheparin plasma was determined by measuring the oleic acid produced by the enzyme-catalyzed hydrolysis of an emulsion containing [$^3$H]-triolein (Amersham TRA 191; Amersham, Buckinghamshire, UK) [17]. The assay mixture containing labeled and unlabeled triolein (Sigma) (1.3 μmol/ml of glyceryl trioleate with a specific activity of 10×106 cpm/μmol) was mixed with 0.11 μmol/ml of L-α-lysophosphatidylcholina (Sigma), 0.2% bovine serum albumin (Sigma) in 0.2 mol/l Buffer Tris–HCl pH 8.8 with NaCl 0.15 mol/l. This mixture was incubated with PHP in saline solution 1:10 and NaCl 1 mol/l for 30 min at 30 °C. After incubation, the reaction was stopped and released fatty acids were isolated by extraction with a carbonate-borate buffer pH=10.5. The released [$^3$H]-oleic acid was quantitated. Results were expressed as μmol/ml h of free fatty acids of postheparin plasma. Using triplicate analysis, the intra-assay CV was 4% and the inter-assay CV 9%.

### 2.8. Lipoprotein isolation and LDL analysis

Lipoproteins were isolated by sequential preparative ultracentrifugation. Total LDL (density=1.019–1.063 g/ml) and dense LDL (1.048–1.063 g/ml) isolation were simultaneously performed from 2 plasma aliquots in an XL-90 Beckman ultracentrifuge, with a type 90 Ti rotor, at 105,000 ×*g* and 15 °C for 18 h. Purity of lipoprotein fractions was tested by agarose gel electrophoresis. Aliquots of isolated LDL were stored at −70 °C for their analyses. Cholesterol, TG (by the methods previously mentioned), phospholipids [18] and total proteins [19] were assessed in isolated LDL fraction and percentual chemical composition was calculated. Cholesterol was measured in dense LDL and this subfraction was expressed as a percentage of total LDL [6].

### 2.9. LDL susceptibility to oxidation

Susceptibility of isolated LDL to oxidation was assessed as previously described [20]. Briefly, after gel filtration chromatography of the lipoprotein fraction on PD-10 desalting columns (Amersham Pharmacia Biotech, Uppsala, Sweden) to remove EDTA and low molecular weight antioxidants, oxidation of LDL (10 mg protein/dl) was promoted in 0.16 mol/l NaCl (pH=7.4), at 37 °C by addition of a freshly prepared $CuCl_2$ solution giving a final concentration of 10 μmol/l. Oxidation blanks were incubated without the addition of copper. LDL oxidation products were assessed by measuring the concentration of thio-barbituric acid reactive substances (TBARS) after 120 min of incubation as previously described [20], and by following the kinetics of conjugated diene formation, continuously monitored by the increase in absorbance at 234 nm [21], in a Hitachi U-1100 spectrophotometer (Tokyo, Japan). *Lag*-time, as an indicator of the resistance to oxidation, was calculated by the intercept of the linear least square slope of the curve with the *x* axis and expressed in min.

Table 1
Subject characteristics and plasma concentration of lipids and lipoproteins

| | PMW $n$=30 | PreMW $n$=28 |
|---|---|---|
| Age, y | 52.9±7 | 33.0±6 |
| Waist circumference (cm) | 86.6±10.1* | 73.9±5.8 |
| BMI (kg/m$^2$) | 26.0±3.5* | 21.4±2.1 |
| Total cholesterol (mmol/l) | 6.34±1.25* | 4.86±0.81 |
| Triglycerides (mmol/l) | 1.56±0.73* | 0.79±0.41 |
| HDL cholesterol (mmol/l) | 1.35±0.31† | 1.56±0.34 |
| LDL cholesterol (mmol/l) | 4.34±1.25* | 2.81±0.81 |
| Apo A-I (mg/dl) | 126±15‡ | 138±24 |
| Apo B (mg/dl) | 120±35* | 79±19 |
| Apo B/ApoA-I | 1.0±0.3* | 0.6±0.1 |

Results are expressed as mean±SD. * $p$<0.0001, † $p$<0.02, ‡ $p$=0.06. PMW: postmenopausal women; PreMW: Pre menopausal women.

Table 2
Comparison of Insulin-resistance markers between pre- and postmenopausal women

| | PMW $n$=30 | PreMW $n$=28 |
|---|---|---|
| Glucose (mmol/l) | 5.34±0.72 | 5.01±0.72 |
| Insulin (μU/ml) | 12.3±8.9* | 6.8±3.1 |
| HOMA | 2.9±2.3* | 1.7±0.7 |
| TG/HDL cholesterol | 2.93±1.82† | 1.25±0.82 |

Results are expressed as mean±SD. *$p$<0.005, † $p$<0.0005. PMW: postmenopausal women; PreMW: Pre menopausal women, TG: triglycerides.

### 2.10. Statistical analysis

Results were expressed as mean±SD for normally distributed data and as median and range for skewed data. Differences between groups were tested using the unpaired Student's *t* test for normally distributed data and the Mann–Whitney *U*-test for skewed data. Correlation between variables were assessed using the Pearson or Spearman correlation tests according to parameter distribution. Multivariate stepwise regression model was developed in order to assess the relationship between modified lipoproteins and other risk factors studied. Differences were considered significant at $p$<5%.

## 3. Results

Overall characteristics for the total sample of women are listed in Table 1. As expected, PMW presented higher body mass index (BMI), waist circumference, TG, total and LDL cholesterol and apo B in comparison to PreMW ($p$<0.001). Besides, the group of PMW showed a decrease in HDL cholesterol ($p$<0.02) and a trend to lower values of apo A-I ($p$=0.06). In addition, the risk index apo B/apo A-I was significantly increased in PMW ($p$<0.0001).

Regarding insulin-resistance markers (Table 2), insulin levels and HOMA were higher in PMW ($p$<0.005). The ratio TG/HDL cholesterol, considered an estimator of insulin-resistance and also of small dense LDL, was significantly increased in women after menopause ($p$<0.0005). Fig. 1-A shows the higher proportion of small dense LDL in this group of women *versus* premenopausal controls ($p$<0.05). LDL percent chemical composition showed a TG enrichment in PMW (9.7±0,6% *versus* 7.4±0.3%, $p$<0.05).

Regarding enzymatic and remodelling factors acting on lipoprotein metabolism, Fig. 1-B shows an increase in hepatic lipase activity in PMW ($p$<0.005), while in Fig. 1-C, a non-significant tendency towards higher values may be observed in CETP from PMW ($p$=0.08).

Table 3 shows the emerging factors related to endothelial injury and inflammation studied in both groups. No differences were found in LDL susceptibility to oxidation. However, Lp-PLA2, homocysteine, hs-CRP, fibronectin and ferritin were detected increased in the postmenopausal group.

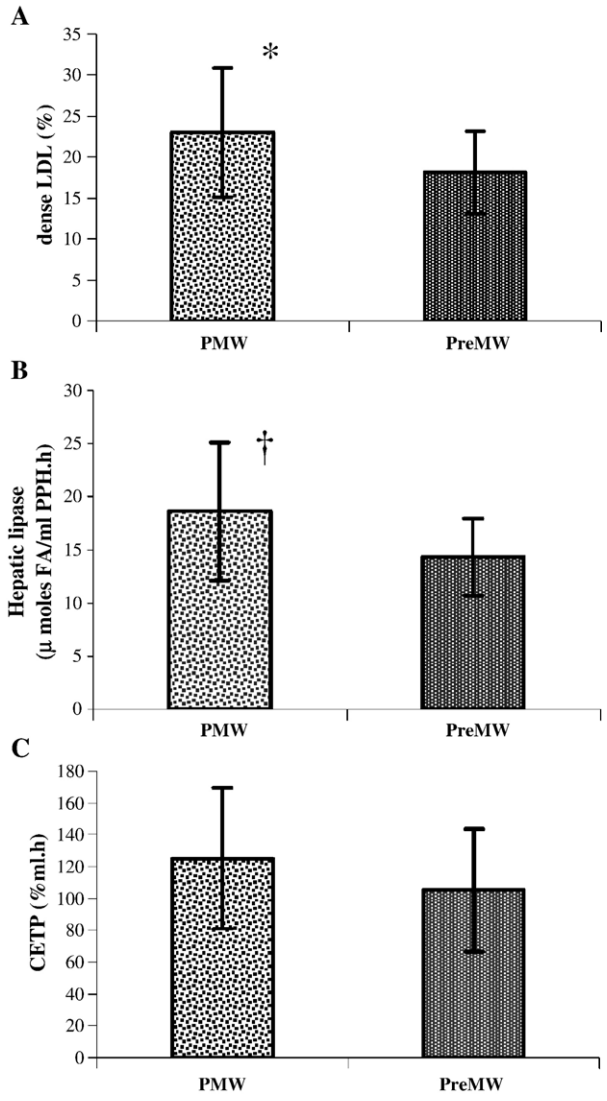


Fig. 1. Small dense LDL proportion (panel A), Hepatic lipase activity (panel B) and CETP (panel C) in premenopausal women (PreMW) and postmenopausal women (PMW).

Table 4 shows the significant correlations obtained between LDL oxidability and/or small dense LDL proportions with the other studied factors. Results revealed that LDL oxidability, expressed as *lag* time, positively correlated with homocysteine, fibronectin, hs-CRP and LDL TG content. Considering LDL-TBARS, significant correlations were found with waist, fibronectin, and small dense LDL percentage. In turn, small dense LDL correlated with insulin-resistance markers and with hepatic lipase activity, but not with CETP activity ($r=0.11$, $p=0.47$). Moreover, this LDL subfraction was associated with the increase of hs-CRP. Regarding ferritin, no correlations were found with LDL oxidability or with other parameters, except with hs-CRP ($r=0.63$, $p<0.00005$). Lp-PLA2 showed a significant association with LDL cholesterol ($r=0.49$, $p<0.001$), but not with HDL ($r=0.12$, $p=0.24$), thus indicating that LDL is its main carrier. Besides, Lp-PLA2 correlated with LDL oxidability measured as LDL-TBARS ($r=0.28$, $p=0.05$). It is noteworthy to mention other significant correlations of hs-CRP with waist ($r=0.52$, $p<0.0006$) and with TG/HDL cholesterol ($r=0.41$, $p<0.004$).

After multiple regression analysis was performed adjusting by menopausal condition, age and waist, LDL-TBARS remained associated with waist ($\beta$: 0.35, $p=0.047$) and small dense LDL ($\beta$: 0.36, $p=0.027$), while the correlation with fibronectin was no longer significant. However, in this model LDL *lag* time maintained the significant inverse associations with fibronectin ($\beta$: −0.41, $p=0.05$), and homocysteine ($\beta$: −0.36, $p<0.038$). Correlation between *lag* time and hs-CRP was lost. When hepatic lipase was added to the model, correlation with small dense LDL remained significant, while associations between this LDL subfraction and the other parameters such as insulin-resistance markers remained not significant. Other interesting finding evidenced after multiple regression analysis was the correlation between Lp-PLA2 and LDL cholesterol ($\beta$: 0.58, $p=0.01$) which indicates that this association is independent of age, menopausal condition and waist. In addition, hs-CRP remained associated with waist after adjustment were assessed ($\beta$: 0.39, $p=0.05$).

## 4. Discussion

In this study, we evaluated the relationship between classic and non-traditional circulating risk factors in hypoestrogenism, reflecting endothelial and subendothelial inflammation and subclinical atherogenic processes. In comparison to PreMW, asymptomatic PMW showed an atherogenic lipoprotein profile, an increase in inflammatory markers and circulating fibronectin coming from the subendothelial space. It is worthy to note a reasonable relationship observed between the above mentioned parameters and the increase in waist circumference, increased hepatic lipase activity, high proportion of small dense LDL and the degree of LDL oxidability.

This study was focused on healthy PMW selected according with the criteria described above, but it must be taken into account that alterations in the lipid–lipoprotein profile began even since the perimenopausal state [22]. Thus, formation of the atherogenic plaque consists of a slow process of evolution,

Table 3
Emerging factors of endothelial injury and inflammation in pre- and postmenopausal women

| | PMW $n=30$ | PreMW $n=28$ |
|---|---|---|
| LDL oxidability | | |
| — LDL TBARS (nmMDA/LDLmg protein) | 55.5±2.8 | 50.5±2.4 |
| — LDL *lag*-time, min | 62±3.3 | 65±1.9 |
| hs-CRP (mg/l) | 3.3* (0.46–12.36) | 1.1 (0.1–7.7) |
| Fibronectin (mg/dl) | 29.6±7.9† | 24.2±6.5 |
| Homocystein (μU/ml) | 12.4±4.4* | 9.1±3.4 |
| Lp-PLA2 (μmol/ml h) | 11.8±2.1† | 9.8±2.8 |
| Ferritin (ng/dl) | 71.3±46.7‡ | 19.3±16.3 |

Results are expressed as mean±SD, except for hs-CRP: median (range) *$p<0.005$, †$p<0.05$, ‡$p<0.0001$. PMW: postmenopausal women; PreMW: Pre menopausal women.

Table 4
Significant correlations between parameters

| Modified LDL | | *versus* | *r* | *p* < |
|---|---|---|---|---|
| LDL oxidability | LDL *lag* time | Homocystein | −0.35 | 0.05 |
| | | Fibronectin | −0.44 | 0.01 |
| | | Hs-CRP* | −0.33 | 0.04 |
| | | LDL-TG | 0.34 | 0.05 |
| | TBARS-LDL | Waist | 0.32 | 0.02 |
| | | Fibronectin | 0.39 | 0.05 |
| | | Small dense LDL | 0.42 | 0.01 |
| Small dense LDL | | HOMA | 0.32 | 0.05 |
| | | TG/HDL cholesterol* | 0.45 | 0.001 |
| | | TG* | 0.41 | 0.04 |
| | | HDL cholesterol | −0.30 | 0.045 |
| | | Waist | 0.36 | 0.02 |
| | | Hepatic lipase | 0.46 | 0.04 |
| | | Hs-CRP* | 0.38 | 0.03 |

Linear regression, * Spearman correlation. TG: triglycerides.

which goes through several steps and takes place since the preclinical state. Unavoidably, there is statistical difference in age between both groups of women because it is difficult to design studies that can separate the effects of the normal aging process from natural menopause. Nevertheless, adjustments for age were performed when associations between variables were evaluated.

Apo B and the apo B/apo A-I ratio were increased in PMW. The former is considered a useful risk marker with high predictive value, since each atherogenic lipoprotein particle contains only one copy of apo B [23]. The ratio of apo B/apo A-I reflects the balance between pro- and antiatherogenic lipoproteins, through their structural apolipoproteins and its ratio has been demonstrated to be a strong predictor of acute myocardial infarction in men and women [24].

Asymptomatic PMW showed higher degree of insulin-resistance, evaluated by means of higher waist circumference, increased HOMA index and the recently proposed ratio of TG/HDL cholesterol, recognized as a useful estimator [25]. The close association between insulin-resistance and menopause has been previously described [26,27].

The small dense LDL proportion was higher in PMW than in PreMW, according to other authors [5]. The high proportion of this atherogenic subfraction leads to an enhanced passage of these particles through the endothelium allowing their binding to proteoglycans, given that small dense LDL have greater affinity than large buoyant LDL [7]. Results from studies carried out in women with clinical and subclinical disease showed that small dense LDL was related to high incidence of coronary heart disease among older women [28].

As previously described, PMW presented high activity of hepatic lipase, presumably due to estrogen decrease plus insulin increase [4]. It is noteworthy that small dense LDL percentage showed a significant positive correlation with the enzyme activity, even after multiple regression analysis, indicating that the increased hepatic lipase would promote small dense LDL formation [29]. Given that no association was found between CETP activity and small dense LDL, the role of hepatic lipase on the generation of these particles must be highlighted. The high proportion of small dense LDL is part of the characteristic atherogenic lipoprotein profile attributed to the associated insulin-resistance state. As a matter of fact, in this study, correlation between small dense LDL and surrogate markers such as waist, HOMA and TG/HDL cholesterol indexes, were observed.

When LDL chemical composition was studied, PMW showed an increased TG content which may stimulate the lipid transfer reaction, resulting in those TG rich and cholesterol ester poor LDL particles. Finally, hepatic lipase hydrolyzes TG in LDL particle, then the size of LDL particles decrease [7,30].

In accordance with previous publications including ours, no differences were found regarding LDL oxidation assessed by its susceptibility to *in vitro* oxidation [31,32]. However, remarkable associations with LDL oxidability were obtained, even after multiple regression analysis. It can be suggested that the higher small dense LDL proportion is, the higher LDL oxidability. Moreover, the higher abdominal fat accumulation measured as waist circumference is, the higher LDL oxidability. Therefore, women with high degree of insulin-resistance would present an increase in small dense LDL proportion and LDL susceptibility to oxidation.

In the present study, Lp-PLA2 activity was higher in PMW than in PreMW. The independent positive correlation of Lp-PLA2 with LDL cholesterol, but not with HDL, suggests that Lp-PLA2 is closely linked to the apo B-containing lipoprotein. This is in accordance with previous results found in hypertriglyceridemic patients [33]. Lp-PLA2 bound to LDL comes from the subendothelial macrophages and its presence in the vascular lumen could be a biomarker of cardiovascular disease [34].

Other emerging factors associated with endothelium damage and a prooxidant and proinflammatory environment are homocystein, hs-CRP, ferritin and fibronectin. Homocysteinemia increases with aging and promotes endothelial dysfunction associated with an impairment in endothelium nitric oxide bioavailability [35]. This could be added to the nitric oxide decrease characteristic of the hypoestrogenism [32]. In accordance with other authors, PMW showed an increase in homocystein [9,35]. It is noteworthy herein that association between homocystein and LDL oxidability remained significant after multivariate adjustment, supporting the already described prooxidant action of homocystein.

Measured with a high sensitive method, hs-CRP also increased in PMW. It is an accepted marker of endothelial inflammation and it has been proposed as an injuring factor belonging to the great number of acute phase proteins [36]. In this study, the median value was over 3.0 mg/l, considered the current cut off value for high risk [37]. The high values of hs-CRP in PMW were associated with LDL oxidability, small dense LDL and waist circumference. The latter was the only parameter which remained linked after multivariate adjustment, suggesting a subclinical inflammatory condition associated to abdominal obesity.

As expected, ferritin plasma concentrations were higher in PMW. However, no association with LDL susceptibility to oxidation was found, in accordance with others [38]. High plasma concentration of ferritin is considered not only a marker

of iron overload, but also an acute phase protein. In a relevant cohort study [39], it was reported that the increase of iron as circulating ferritin is a risk factor for stroke, with higher hazard figures in menopausal condition. The close association obtained in this study between ferritin and hs-CRP supports the concept that both of them belong to the cluster of proinflammatory proteins present in menopausal condition.

The passage of fibronectin through the endothelial barrier to the blood would be another marker of a preclinical atherogenic process and endothelial injury in menopause. An increase in circulating fibronectin in PMW was observed and it was associated with LDL oxidability, even after multiple regression analysis. An interpretation can be that oxidized LDL may damage the endothelium, promoting the release of subendothelial extra cellular matrix components such as fibronectin and metalloproteases among others.

There are few publications regarding the interaction among abnormal LDL, inflammatory, oxidative stress and insulin-resistance markers and subendothelial extracellular matrix components in apparently healthy women in postmenopausal condition. As a whole, alterations observed in this study would evidence a preclinical active atherosclerotic process in PMW.

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