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Rev Inves Clin. 2015;67:122-9 ORIGINAL ARTICLE

Menopausal Women Have Hypofibrinolysis Even in Subclinical Stage of Atherosclerosis

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ABSTRACT

Background: PAI-1 is the main inhibitor of fibrinolysis. Increase in PAI-1 levels has been associated with the risk of coronary disease; however, there are few studies on the relationship between subclinical atherosclerosis and PAI-1 levels. **Objective:** The aim of this study was to analyze the relationship between PAI-1 level and carotid intima-media thickness in premenopausal and postmenopausal women without apparent cardiovascular disease. **Material and Methods:** A cross-sectional study was conducted in 142 women aged 45 to 60 years with no history of cardiovascular disease. Anthropometric and laboratory measurements were performed, including PAI-1 levels. All participants underwent a B-Mode ultrasound to measure intima-media thickness. Subclinical atherosclerosis was considered when intima-media thickness was ≥ 0.7 mm and/or an atheromatous plaque was observed. **Results:** Postmenopausal women had greater intima-media thickness than premenopausal women (0.688 ± 0.129 vs. 0.621 ± 0.113 mm; $p < 0.05$). Compared to women with normal intima-media thickness, women with subclinical atherosclerosis had higher PAI-1 levels (23.2 ± 13.7 vs. 30.4 ± 20.7 ng/ml; $p < 0.05$). In all participants, intima-media thickness correlated with PAI-1 ($r = 0.302$; $p = 0.01$) and with age ($r = 0.358$; $p = 0001$). **Conclusions:** An increase in intima-media thickness was observed in postmenopausal women compared with premenopausal women. Asymptomatic women with increased intima-media thickness had higher PAI-1 levels. These findings suggest that fibrinolytic activity is low in the subclinical stage of atherosclerosis.
(REV INVES CLIN. 2015;67:122-9)
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Key words: Atherosclerosis. Fibrinolysis. Menopause. Carotid intima-media thickness.

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Received for publication: 29-07-2014
Accepted for publication: 11-12-2014

BACKGROUND

The risk of cardiovascular disease increases considerably in women after the age of 50[1]. This increased risk has been explained by an estrogen decline associated with redistribution of body fat to an intra-abdominal predominance, changes in serum lipid profile, and modifications in hemostasis that lead to a prothrombotic state, among other factors[2-4].

One of the hemostatic changes that can be observed in postmenopausal women is a decrease in fibrinolytic activity due to an increase in plasminogen activator inhibitor-1 (PAI-1) levels[2,5-6]. Several studies have found a relationship between coronary disease and an increase in PAI-1 levels[7,8]. Thus, elevated PAI-1 has been considered a marker of cardiovascular risk (CVR) and a prognostic indicator for acute myocardial infarction[2,5,9].

The expression of PAI-1 has been observed in arteries that are affected by atherosclerosis, which suggests its participation in the pathogenesis and progression of atherosclerotic disease[10-13]. Atherosclerosis is a degenerative process featuring growth of the intima-media layers[14,15]. Studies have demonstrated that intima-media thickness (IMT) of the carotid artery measured by ultrasonography is useful as an independent predictor of cardiac events[16,17].

Increased PAI-1 levels have been found in patients with clinical manifestations of atherosclerotic disease[18,19]. Another study found a relationship between IMT and PAI-1 levels in young adults[20]. However, there is little information regarding PAI-1 levels in menopausal women with subclinical atherosclerosis.

OBJECTIVE

The main objective of this study was to analyze the relationship between PAI-1 plasma levels and IMT of the carotid artery in pre- and postmenopausal women without apparent cardiovascular disease.

MATERIAL AND METHODS

Study population

We studied 270 women aged 45 to 60 years, without apparent cardiovascular disease, and who consecutively attended the Medical Research Unit in Endocrine Diseases of the National Medical Center, Mexican Social Security Institute (Unidad de Investigación Médica en Enfermedades Endocrinas, Centro Médico Nacional, IMSS). Exclusion criteria were clinical data or history of cardiovascular disease, diabetes, hepatic and/or renal failure, endocrine disease, or hematological disease. Participants who were found to be under hormonal treatment or receiving any other medication that could affect hemostatic function were also excluded. A total of 142 women who met the inclusion criteria were ultimately selected for this study. The study was approved by the Ethics Committee of the Mexican Social Security Institute. Subjects who agreed to participate signed an informed consent form.

Participants underwent a clinical examination; body weight, height, and arterial pressure were measured. Body mass index (BMI) was calculated by dividing the weight by the square of the height (kg/m^2). With the participant standing, the waist circumference was measured at the midpoint of the distance between the iliac crest and the inferior border of the last rib.

Menopausal state

The diagnosis of menopause was established based on a history of amenorrhea of one year or more, estradiol concentration < 20 pg/ml, and follicle-stimulating hormone concentration > 30 mIU/ml.

Intima-media thickness assessment

To measure IMT, participants were placed in the supine position with slight neck extension. Both common carotid arteries were assessed using pulsed color Doppler. The average thicknesses of the intima-media layer of the posterior wall of the right and left common carotid arteries and the existence of atheromatous plaques were evaluated according to the Mannheim Consensus[21]. The study was completed using a high-resolution B-mode ultrasonography device (Aloka-α7 multifrequency linear transducer). All measurements were taken by the same operator. Participants with an IMT of < 0.7 mm were considered normal. Subclinical atherosclerosis was considered when the IMT was ≥ 0.70 mm and/or atheromatous plaques were observed[22].

Laboratory Procedures

Peripheral venous samples were taken from participants at 8:00 AM after a fast of ≥ 10 hours. Each

blood sample was collected in two tubes, one containing sodium citrate and the other without anticoagulant. Samples were centrifuged at 3,000 rpm for 15 minutes at 4ºC, and aliquots of plasma and serum were prepared for testing. Aliquots for measuring PAI-1 and insulin levels were stored at –70ºC until assayed. Plasma concentrations of PAI-1 were measured by enzyme-linked immunosorbent assay (BioVendor, USA). Serum insulin and estradiol were measured using radioimmunoassay (Millipore, Billerica, MA, USA).

Determination of other cardiovascular risk factors

Serum glucose, high-density lipoprotein (HDL) cholesterol, and triglyceride levels were determined using enzymatic methods with Spinreact reagents. The insulin resistance index was calculated using HOMA (homeostasis model assessment): insulin (mU/ml) × glucose (mmol/l)/22.5[23].

Genomic DNA was obtained from peripheral blood using a commercial kit (Invitek GMBH, Berlin, Germany) according to the manufacturer's instructions. Genotyping of the 4G/5G polymorphism in the PAI-1 promoter region was performed by PCR using the following oligonucleotides: 5'-CACAGAGAGAGTCTGGCCACGT-3' (sense) and 5' CCAACAGAGGACTCTTGGTCT-3' (antisense). Amplification products of 99 bp (5G) and 98 bp (4G) were obtained[24]. Products were subjected to digestion with specific restriction enzyme *Bs*I-1 (Fermentas, Life Sciences, Hamilton ON, Canada). The DNA fragments were separated by electrophoresis in 2% agarose gels and visualized using ethidium bromide.

The diagnosis of metabolic syndrome was established according to the International Diabetes Federation (IDF) definitions for clinical practice[25,26]. Criteria were waist circumference ≥ 80 cm in addition to two of the following: fasting glucose ≥ 100 mg/dl, triglycerides ≥ 150 mg/dl, HDL cholesterol < 50 mg/dl or taking lipid-lowering medications, systolic pressure ≥ 130 and/or diastolic pressure ≥ 85 mmHg, or taking antihypertensive medications.

Statistical analysis

The qualitative variables are expressed as frequencies and the quantitative variables, as mean ± standard deviation (M ± SD). Mann-Whitney test was used to compare quantitative measurements. Spearman test was used to establish correlations between PAI-1, carotid IMT, and other variables. A multivariate regression model was generated to determine the influence of anthropometric, metabolic, and hormonal variables on IMT. Values of $p \leq 0.05$ were considered significant. Analyses were performed using SPSS, version 20.0.

The sample size of this study had sufficient statistical power to identify a correlation between IMT and PAI-1 of at least 0.30 and to detect a difference in the concentration of PAI-1 of ≥ 25% between the groups with normal and increased IMT.

RESULTS

The mean age of participants was 52 ± 5.4 years; 69.7% were found to be in the postmenopausal stage (Table 1).

Menopausal State

Compared with premenopausal women, postmenopausal women had higher waist circumference (91.9 ± 11.5 vs. 87.1 ± 9.2 cm; $p < 0.05$), BMI (28.1 ± 4.8 vs. 27.0 ± 3.9; $p < 0.05$), glucose levels (89.1 ± 24.0 vs. 80.2 ± 12.5 mg/dl; $p < 0.05$), insulin resistance (4.8 ± 3.35 vs. 3.12 ± 1.52) and IMT (0.688 ± 0.129 vs. 0.621 ± 0.113 mm; $p = 0.014$). Other factors including blood pressure and smoking were similar in both groups (Table 1).

Intima-media thickness and PAI-1

When the population was analyzed according to IMT, 79 participants (55.6%) were classified in the subclinical atherosclerosis group, 10 of whom had atherosclerotic plaque. The PAI-1 levels were higher in women with subclinical atherosclerosis compared to women with normal IMT (30.4 ± 20.7 vs. 23.2 ± 13.7 ng/ml; $p < 0.05$) (Fig. 1). Women with subclinical atherosclerosis also had an increase in other CVR factors, including glucose, triglycerides, low-density lipoprotein (LDL)-cholesterol and C-reactive protein levels as well as increased insulin resistance; however, only age was significantly different (Table 2).

Analysis of the correlation between IMT, PAI-1, and the metabolic variables is shown in table 3. A positive

Table 1. Characteristics of the study participants

Characteristics	Premenopausal (n = 43)	Postmenopausal (n = 99)	Total (n = 142)
Age (years)	46.6 ± 2.1	54.8 ± 5.5	52.5 ± 5.4
BMI (kg/cm^2)	27.0 ± 3.9	28.1 ± 4.8	28.1 ± 4.8
Waist circumference (cm)	87.1 ± 9.2	91.9 ± 11.5*	90.9 ± 11.5
Systolic blood pressure (mm/Hg)	107.5 ± 15.6	112.2 ± 14.5	110.9 ± 14.8
Diastolic blood pressure (mm/Hg)	72.2 ± 8.4	75.2 ± 9.3	74.5 ± 9.3
Fasting glucose (mg/dl)	80.2 ± 12.5	89.1 ± 24.0*	86.9 ± 21.9
Current smoker n (%)	3 (6.9)	7 (7.1)	10 (7.0)
Total cholesterol (mg/dl)	208.8 ± 37.3	241.9 ± 52*	235.7 ± 50.4
Triglycerides (mg/dl)	155.2 ± 85.3	181.2 ±119.1	173.2 ± 108.1
HDL-cholesterol (mg/dl)	56.6 ± 10.7	54.2 ±14.5	55.2 ± 14.0
HOMA-IR	3.12 ± 1.52	4.8 ± 3.35†	4.35 ± 3.10
Insulin (mUI/ml)	15.5 ± 7.2	21.3 ± 12.8†	19.7 ± 11.5
PAI-1 (ng/ml)	26.6 ± 21.0	28.0 ± 18.1	27.0 ± 16.0
IMT (mm)	0.621 ± 0.113	0.688 ± 0.129*	0.667 ± 0.126

*The p value represents the difference between premenopausal and postmenopausal women; *$p < 0.05$, $^{\dagger}p < 0.01$.
BMI: body mass index; HOMA-IR: insulin resistance index determined by Homeostasis Model Assessment; PAI-1: plasminogen activator inhibitor type-1; IMT: intima-media thickness of the carotid artery.

correlation was found between IMT and PAI-1, as well as with glucose levels and insulin resistance. The relationship between IMT and the concentration of estradiol was negative ($r = –0.181$; $p = 0.04$), which was even more evident in postmenopausal women ($r = –0.326$; $p = 0.04$).

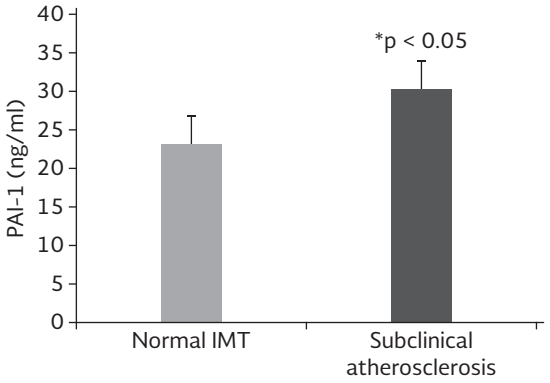
PAI-1 and other cardiovascular risk factors

Of all the participants, 47 (33.1%) had metabolic syndrome. Consequently, compared with women without metabolic syndrome, this group had greater central obesity (95.5 ± 10.8 vs. 88.6 ± 11.6; $p < 0.01$), higher glucose (97.3 ± 19.8 vs. 81.7 ± 10.1; $p < 0.01$), triglycerides (239.2 ± 42.6 vs. 140.4 ± 65.4; $p < 0.01$), blood pressure (116.0 ± 16.4/76.7 ± 9.8 vs. 108.3 ± 13.2/73.4 ± 9.8; $p < 0.01$), and insulin resistance (6.31 ± 4.42 vs. 3.44 ± 1.66; $p < 0.0001$), and lower HDL cholesterol (45.1 ± 11.2 vs. 60.1 ± 12.5; $p < 0.01$).

The concentration of PAI-1 was higher in women with metabolic syndrome, although the difference was not statistically significant. However, in the group of premenopausal women, a significant increase in PAI-1 was found among women with metabolic syndrome (49.9 ± 13.5 vs. 24.5 ± 14.4; $p < 0.01$). Other findings were the correlation of PAI-1 with BMI ($r = 0.227$; $p = 0.02$), waist circumference ($r = 0.182$; $p < 0.05$), and insulin resistance ($r = 0.425$; $p < 0.0001$).

A multiple regression analysis was performed to estimate which variables could predict IMT measures. The independent variables included were age and menopausal state as well as estradiol, blood pressure, triglyceride, total cholesterol, HDL cholesterol, glucose, insulin resistance, 4G/5G polymorphism, and PAI-1 levels. Age was the variable with the greatest influence on IMT ($\beta_{standardized} = –0.117$; $p < 0.05$). After adjusting for age, it was found that, in addition to PAI-1, the concentration of estradiol and the degree of insulin resistance influenced IMT ($\beta_{standardized} = 0.0223$; $p < 0.05$).

Figure 1. Concentration of PAI-1 in women with normal intima-media thickness of the carotid artery and subclinical atherosclerosis as determined by an increased intima-media thickness.



PAI-1: plasminogen activator inhibitor-1; IMT: intima-media thickness.

Table 2. Characteristics of participants with normal IMT and subclinical atherosclerosis

Characteristics	Healthy (n = 63)	Subclinical atherosclerosis (n = 79)	p
Age (years)	50.4 ± 4.2	54.2 ± 6.5	**0.0001**
BMI (kg/cm^2)	28.2 ± 4.9	28.1 ± 4.7	NS
Waist circumference (cm)	90.7 ± 11.0	91.2 ± 12.0	NS
Systolic blood pressure (mmHg)	110.1 ± 13.6	111.5 ± 15.7	NS
Diastolic blood pressure (mmHg)	74.6 ± 9.2	74.3 ±.9.5	NS
Fasting glucose (mg/dl)	83.3 ± 14.8	89.8 ± 26.0	0.051
Total cholesterol (mg/dl)	229.3 ± 53.6	239.6 ± 48.8	NS
Triglycerides (mg/dl)	162.6 ± 111.5	181.8 ± 105.3	NS
HDL-cholesterol (mg/dl)	56.7 ± 12.8	54.0 ± 14.8	NS
LDL-cholesterol (mg/dl)	142.5 ± 45.8	151.2 ± 44.0	NS
C-reactive protein (mg/l)	3.2 ± 2.4	4.0 ± 2.9	NS
HOMA-IR	3.8 ± 2.0	4.7 ± 3.7	0.07

The p value represents the difference between women with subclinical atherosclerosis and healthy.
BMI: body mass index; HOMA-IR: insulin resistance index determined by Homeostasis Model Assessment; NS: non significant.

DISCUSSION

In this study, the most important finding was a direct correlation between circulating levels of PAI-1 and the IMT of the carotid artery in women older than 45 years without clinical evidence of cardiovascular disease.

PAI-1 is an inhibitory protein of the fibrinolytic system that is mainly synthesized in the vascular endothelium, in addition to the liver, adipose tissue, and platelets[27]. The PAI-1 likely plays an important role in the atherogenesis process[28,29]. Some clinical studies have confirmed increased levels of PAI-1 in patients with established atherosclerotic disease[18,19,30,31]. The increase in circulating PAI-1 produces a state of hypofibrinolysis and an increase in fibrin deposits in the atherosclerotic plaque[18]. Additionally, PAI-1 that is located in the atherosclerotic plaque regulates cellular migration and adhesion[30-32]. Its overexpression at this site can lead to the formation of an occlusive thrombus[27,33] and indicates changes that render the plaque vulnerable and at greater risk for rupture[34-37].

Table 3. Correlation between carotid artery intima-media thickness and clinical and laboratory data

	r	p
Age	0.358	0.0001
BMI	–0.052	NS
Waist circumference	0.030	NS
Blood pressure		
– Systolic	0.026	NS
– Diastolic	0.010	NS
Fasting glucose	0.147	0.04
Total cholesterol	–0.168	NS
Triglycerides	-0.036	NS
HDL-cholesterol	0.017	NS
LDL-cholesterol	0.135	0.06
Estradiol	–0.181	0.04
C-reactive protein	0.106	NS
Insulin	0.079	NS
HOMA-IR	0.188	0.04
PAI-1	0.302	0.01

BMI: body mass index; HOMA-IR: insulin resistance index determined by Homeostasis Model Assessment; PAI-1: plasminogen activator inhibitor type-1; IMT: intima-media thickness of the carotid artery; NS: non significant.

Previous studies have shown that patients with symptomatic atherosclerosis and myocardial infarction have elevated PAI-1. In our study, we also found that menopausal women have increased PAI-1 levels even in the subclinical stage of atherosclerosis. Similarly, but in another physiopathological context, patients with primary antiphospholipid antibody syndrome also have increased PAI-1 and IMT[38]. This suggests that the increased thrombotic risk in both clinical entities could be at least partially explained by decreased fibrinolytic activity through increased PAI-1.

In our study, the diagnosis of subclinical atherosclerosis was made by measuring the IMT of the carotid artery and following criteria from the Mannheim Consensus for the early identification of atherosclerosis and the Latin American study CARMELA[23,24]. Measuring IMT

of the carotid artery is useful for the detection of both the subclinical stages and already established atherosclerosis[37-41]. In a meta-analysis, it was demonstrated that an increase of 0.1 mm in the IMT elevates the future risk of myocardial infarction by 10-15% and of cerebral vascular disease by 13-18%[41,42]. Therefore, the identification of subclinical atherosclerosis using IMT could be of great use in stratifying high-risk groups, such as postmenopausal women with other associated CVR factors. This information will support the implementation of strategies for the prevention of cardiovascular events. Nonetheless, further studies are needed to identify the reference values for IMT in specific populations and to identify patients with increased CVR. To date there has been no global consensus on the protocols for measuring IMT of the carotid artery and on which arterial segment it should be evaluated[43].

Another observation from this study is that there is an increase in IMT in postmenopausal compared with premenopausal women, which could be interpreted as an increase in CVR in the former group. This observation is similar to previous studies that found an increased IMT in postmenopausal compared to premenopausal women[44,45]. The greater CVR in postmenopause is related to an estrogen decline and to other factors including endothelial dysfunction[46]. This finding supports the concept that estrogen deficit contributes to the development of early atherosclerosis[47]. Nevertheless, it is debated whether menopause is an independent determinant of IMT because other studies have suggested a stronger correlation with age[40,48]. Aging is considered an independent determinant of increased CVR[49,50] and it has been found that carotid artery IMT increases by 0.01 to 0.02 mm for each year of life[50].

Our results also suggest that increases in IMT and PAI-1 could be influenced by other elements such as insulin resistance; in turn, insulin resistance is related to other factors including menopause, aging, and hyperlipidemia[51]. Insulin plays an important role in the maintenance of blood vessel homeostasis, and the presence of insulin resistance can be one of the connections between different risk factors that affect endothelial function[52-54].

We found that premenopausal women with metabolic syndrome had higher PAI-1 levels compared to those without the syndrome. In postmenopausal women this difference was not significant. Probably due to the characteristics of this group, such as increase of BMI, central obesity, and insulin resistance, we could not observe differences in PAI-1 levels. Such factors are determinants on PAI-1 concentrations[51]. Previous studies have shown that obese subjects with insulin resistance, but without metabolic syndrome comorbidities, have high PAI-1 levels[55]. It has also been proposed that PAI-1 acts as a causative factor in the development of the metabolic syndrome[56].

In this study we did not find an association between IMT and 4G/5G polymorphism of the PAI-1 gene, a result that is similar to other studies[57]. However, further studies are needed in our population with a larger sample size to rule out a possible association of this polymorphism with carotid artery IMT.

One limitation of our study is its cross-sectional design, which prevents the demonstration of causality between circulating PAI-1 levels and carotid artery IMT. Data from longitudinal studies are needed to establish the mechanism by which circulating levels of PAI-1 affect the atherosclerosis process because it is still not known whether circulating PAI-1 leads to vascular damage, or if vascular changes cause an increase in PAI-1.

To our knowledge, this is the first study in postmenopausal women that describes changes in fibrinolysis during the subclinical stage of atherosclerosis. According to cross-sectional and longitudinal studies, an increase in carotid IMT precedes the appearance of atherosclerotic plaque[41,58]. Thus, the identification of increased IMT could be relevant in postmenopausal women due to the higher incidence of cardiovascular disease in this stage of life.

In summary, in this study of asymptomatic women without apparent cardiovascular disease, carotid artery IMT was increased in postmenopausal compared with premenopausal women. A positive correlation was found between IMT and circulating levels of PAI-1. Data suggest that hypofibrinolysis is present even in the subclinical stage of atherosclerosis. This observation could be relevant for the prevention of cardiovascular disease during menopause, a state with greater risk of thrombotic events.

ACKNOWLEDGMENTS

This study was supported by Consejo Nacional de Ciencia y Tecnología (CONACYT) of Mexico, project 2013-1-201874.

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