# Effects of Estrogen on Vascular Inflammation
## A Matter of Timing

Susana Novella, Magda Heras, Carlos Hermenegildo, Ana Paula Dantas

***Objective***—Our study aims to determine the role of time of menopause on vascular inflammation biomarkers and how it affects their modulation by estrogen and raloxifene in postmenopausal women.

***Methods and Results***—Uterine arteries from 68 postmenopausal women were divided into 3 segments and cultured for 24 hours in tissue culture media containing 17β-estradiol (100 nmol/L), raloxifene (100 nmol/L), or vehicle. Assessment of arterial concentration of 13 inflammatory biomarkers was performed by multiplex immunobead-based assay. Aging per se has a positive correlation with the generation of several proinflammatory markers. Although short-term estradiol exposure correlates with lower expression of tumor necrosis factor-α, vascular endothelial growth factor, and interleukin-1β in all age groups, for most biomarkers aging was associated with a switch from a beneficial anti-inflammatory action by estrogen, at earlier stages of menopause, to a proinflammatory profile after 5 years past its onset. Raloxifene has no significant effect on the expression of all proinflammatory markers. Western blot analysis of estrogen receptor expression (estrogen receptor-α and estrogen receptor-β) showed that estrogen receptor-β increases with aging, and this increase has a positive correlation with the generation of several proinflammatory markers.

***Conclusion***—Aging alters estrogen-mediated effects on the modulation of inflammatory biomarkers in women. How aging affects estrogen responses on vascular inflammation is not clear, but our data show a positive association between increased estrogen receptor-β expression with aging and proinflammatory effects by estrogen. **(*Arterioscler Thromb Vasc Biol.* 2012;32:2035-2042.)**

**Key Words:** vascular inflammation ■ menopause ■ estrogen ■ raloxifene ■ multiplex immunoassay



Vascular inflammation and endothelial dysfunction are key elements in the progression of cardiovascular disease (CVD). In most conditions that are considered cardiovascular risk factors (eg, hypercholesterolemia and hypertension), the vascular system or, more specifically, vascular endothelium incites an inflammatory response that leads to the development of adherent and dysfunctional endothelial cell, which in turn contributes to vascular damage and risk for CVD.[1]

In women, the processes involved in vascular damage are thought to be slowed by the presence of estrogen.[2] Nevertheless, available data on the effects of estrogens on inflammatory process are fairly contradictory, with both anti-inflammatory[3,4] and proinflammatory[5] effects reported. This is further complicated by discrepant data from prospective randomized clinical studies aimed to determine the effects of estrogen replacement therapy on circulating markers of inflammation in postmenopausal women. These studies described that biomarkers such as adhesion molecules and interleukins (ILs) can be increased, decreased, or unchanged after estrogen treatment.[4,6,7]

Type of estrogen and cell/tissue studied, acute or chronic stimuli, degree of tissue injury, and differences in end points used to define the inflammatory response are some of the factors that may explain these discrepancies. In addition, there is a recent consensus that cardiovascular protective effects of estrogen may be influenced by the timing of initiation of estrogen therapy. A recent theory, called timing hypothesis, has postulated that the effects of estrogen to prevent CVD may occur only when treatment is initiated before the detrimental effects of aging on CVD are established in the vasculature.[8] Aging, per se, is known to have a positive relationship with the levels of inflammation biomarkers and increased risk of CVD.[9] Nonetheless, it is currently unknown whether the effects of estrogen on vascular function and, more specifically, vascular inflammation are modified by aging in women. In this regard, the present study aims to evaluate the relationship of aging with vascular inflammation biomarkers and how time since menopause affects the modulation of inflammatory biomarkers by estrogen and raloxifene in arteries of postmenopausal women.

## Methods

The protocol was approved by the Ethics Committee of Clinical Research (Comite Etico de Investigacion Clinica Protocol 2007/3916) from Hospital Clinic de Barcelona and conducted in compliance with

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the principles of the Helsinki Declaration. Uterine arteries were obtained from 68 women who underwent hysterectomy because of uterine prolapse or myoma from September 2007 to June 2009. All patients provided written informed consent to participate in the study. Women who have never been on hormone replacement therapy or selective estrogen receptor modulators (raloxifene and tamoxifen) were matched for body mass index, blood pressure, lipids, and lipoproteins. Exclusion criterion includes use of chronic anti-inflammatory therapy, statins, angiotensin-converting enzyme inhibitors, and angiotensin receptor type 1 antagonist. At the moment of hysterectomy, arteries were cleaned, divided into 3 segments, and cultured for 24 hours in tissue culture media containing 17β-estradiol (100 nmol/L), raloxifene (100 nmol/L), or vehicle for further analysis of inflammation biomarkers and estrogen receptor (ER) expression. Detailed methods related to tissue culture and treatments, multiplex assay, and Western blot are shown in the online-only Data Supplement.

## Data Analysis

Time since menopause was defined by the age at which a woman last had any menstrual bleeding and expressed as years passed since last menstruation until the day of hysterectomy. Inflammation biomarker concentration in each sample was expressed as mean concentration from triplicate measurements normalized by the amount of protein in each sample. The degree of linear relationship between time since menopause and biomarker concentration found in each group (ie, estrogen-treated, raloxifene-treated, and untreated groups), as well as the correlation between ER-α or ER-β expression (in untreated arteries) and biomarker concentration after treatments (estrogen or raloxifene), was expressed as regular linear regression compared with the use of Pearson correlation coefficient, expressed as Pearson *r* value, ranging from −1 (inverse correlation) to +1 (positive correlation). The proportion of variation response shared by the 2 variables in the model is expressed as $r^2$. Significant correlation was accepted at $P<0.05$. ANCOVA was used to compare the slopes of correlations from untreated arteries with the slopes obtained by different treatments (17β-estradiol or raloxifene). The magnitude of estrogen and raloxifene effects on inflammation biomarker concentration in association with time since menopause was performed by combination of samples in age groups as follows: menopausal women up to 5 years before hysterectomy (<5); women between 5 and 10 years past the onset of menopause (5–10); and women >10 years past menopause (>10). Association between time since menopause in grouped samples and estrogen or raloxifene treatments was assessed using 2-way ANOVA, followed by Bonferroni post-test to compare replicate means. Statistical analysis and Pearson coefficient calculation were carried out using SPSS Statistics v18.0 software (IBM, Armonk, NY).

## Results

Clinical characteristics of women who provide uterine arteries are presented in the Table. Despite all care in obtaining samples from patients without history of CVD, a few women in this study have presented some degree of cardiovascular risk factors, including hypertension and hypercholesterolemia, which were equally distributed among aging groups. No racial/ethnic-based evaluation was performed.

Aging per se has a positive correlation with vascular inflammation. Eight of 13 markers displayed a sizeable positive relationship between time since menopause and inflammatory biomarkers (Table I in the online-only Data Supplement). ANCOVA revealed that short-term estradiol exposure significantly affects aging-associated correlation observed in most biomarkers (Figure 1). Estrogen treatment changed the degree of linear correlation of inflammatory biomarkers in association with time since menopause, although these effects were not uniform for all markers studied (Figure 1). Although estrogen treatment was associated with a reduction in the concentration of IL-1β, vascular endothelial growth factor, and tumor necrosis factor (TNF-α) at any stage of menopause, an increased correlation was observed for monocyte chemotactic protein-1 and the adhesion molecules soluble intercellular adhesion molecule-1 and soluble vascular cell adhesion molecule-1. In addition, treatment with estrogen created a correlation that had not previously existed between the production of IL-6 and IL-8 and time of menopause (Figure 1). Raloxifene had no significant effects on the relationship of time since menopause with all proinflammatory markers studied (Table I in the online-only Data Supplement). When arteries were grouped by time after menopause, we observed a bidirectional effect of estrogen. Although estrogen treatment was associated with lower expression of the markers TNF-α, vascular endothelial growth factor, and IL-1β in all age groups (Figure 2), for most of the inflammation biomarkers, time of menopause was associated with a switch from a beneficial anti-inflammatory action by estrogen, at earlier stages of menopause, to a proinflammatory profile after 5 years past its onset (Figure 3). Western blot analysis of ER-α expression in nontreated arteries reveals the presence of 2 splicing variants of this receptor in the arterial wall, the 66-kDa (ER66) and the 46-kDa (ER46) isoforms (Figure 4A). Time since menopause does not modify the expression of ER66, whereas a slight, although not significant, increase in the ER46 isoform was observed in the >10-year group (Figure 4A). Analysis of ER46/ER66 ratio was found to have no correlation with time since menopause (Figure 4C). On the other hand, although ANOVA showed no statistical significance among aging groups (ER46/ER66 ratio: <5 group, 0.57±0.03; 5–10 group, 0.63±0.05; >10 group, 0.70±0.07), a significant aging-associated linear trend increase in ER46/ER66 was observed (ANOVA post-test for linear

**Table. Baseline Demographic and Clinical Characteristics**

| Characteristics | |
|---|---|
| Age, mean (SD) | 58 (13) |
| Time since menopause (y), n (%) | |
| <5 | 30 (44) |
| 5–10 | 17 (25) |
| >10 | 21 (31) |
| Reason for hysterectomy, n (%) | |
| Myoma | 39 (57) |
| Uterine prolapse | 29 (43) |
| BMI, mean (SD) | 28 (3.5) |
| Total cholesterol (mg/dL), mean (SD) | 212 (22) |
| Triglycerides (mg/dL), mean (SD) | 91 (19) |
| History of CVD, n (%) | |
| Hypertension | 14 (21) |
| Stroke | 0 (0) |
| Angina | 0 (0) |
| Smoking, n (%) | |
| Never | 54 (80) |
| Past | 9 (13) |
| Current | 5 (7) |

BMI indicates body mass index; CVD, cardiovascular disease.

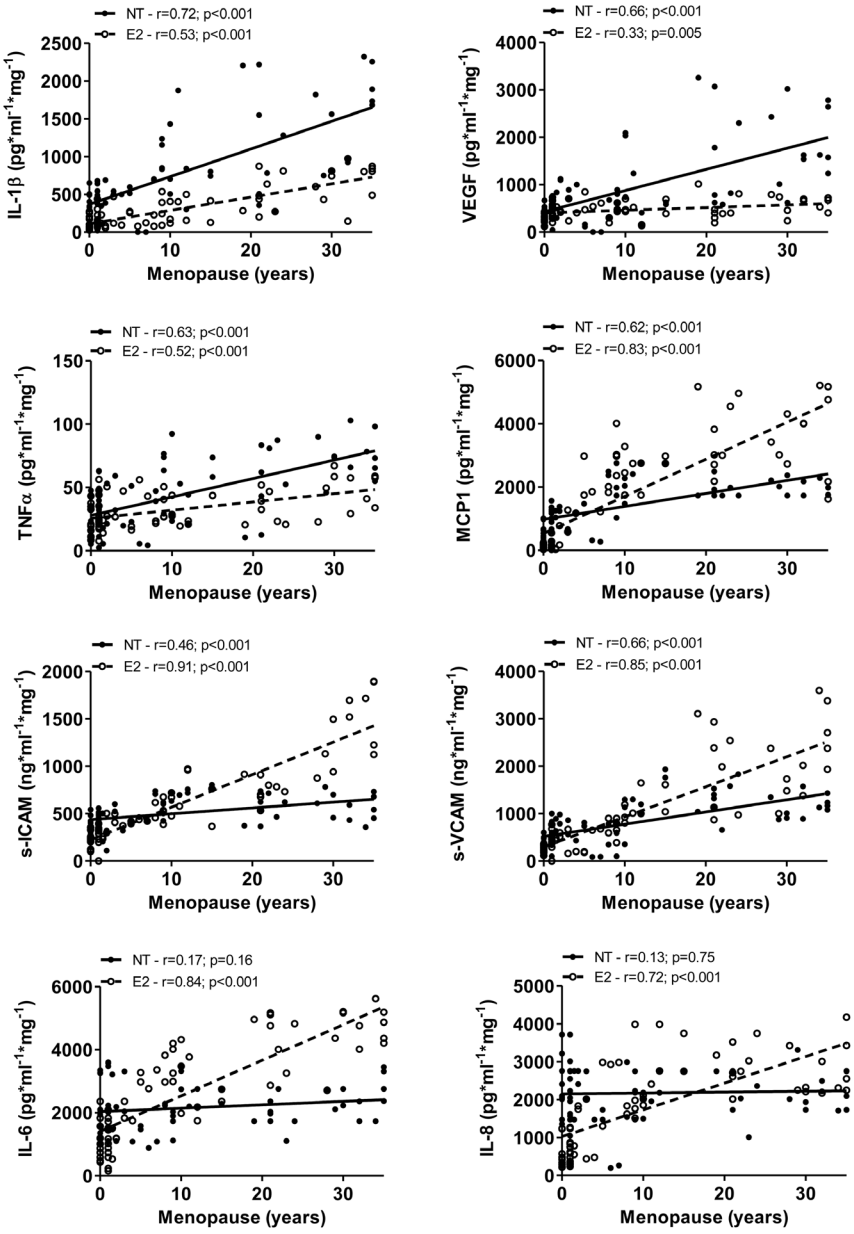


**Figure 1.** Simple correlation analysis between time since menopause (*x* axis) and concentration of biomarkers of inflammation (*y* axis) in uterine arteries untreated (NT) and treated with 100 nmol/L 17β-estradiol (E2). Shown are the inflammatory biomarkers that expressed significant changes in the degree of correlation after E2 treatment, as determined by ANCOVA. TNF-α indicates tumor necrosis factor-α; VEGF, vascular endothelial growth factor; IL, interleukin; MCP1, Monocyte chemotactic protein-1; s-ICAM, soluble intercellular adhesion molecule-1; s-VCAM, soluble vascular cell adhesion molecule-1.

trend: *P*=0.0075). As for ER-β, we observed a linear trend of increase in ER-β expression with aging (ANOVA post-test for linear trend: *P*=0.013), which becomes significantly higher past 10 years of menopause onset (Figure 4B). A similar pattern of responses on ER-α and ER-β expressions was found in arterial segments that were treated with 17β-estradiol or raloxifene (data not shown), suggesting that there is no modulation of ER expression by these agonists in our experimental conditions (24 hours of treatment at 100 nmol/L). By correlating the expression of ERs with arterial biomarkers of inflammation, no association was found between these markers and ER-α expression (Figure I in the online-only Data Supplement). However, a significant positive relationship was observed between ER-β expression and the proinflammatory effects of estrogen (Figure 5). Interestingly, we also found a lower, but positive, correlation between the expression of ER-β and the concentration of TNF-α and IL-1β, 2 biomarkers that were found to be downregulated by estrogen (Figure 5).

## Discussion

Our data are a first step toward the understanding the effects of aging, or rather time since menopause, in the modulation of vascular inflammation by estrogen. Based on the principle of the timing hypothesis, the anti- or proinflammatory potential of 2 estrogenic molecules (17β-estradiol and raloxifene) was carefully determined by treating arterial segments from women at different stages of menopause.

Together with the known risk factors, findings from epidemiological and basic studies have tightly linked inflammatory process with vascular aging.[10,11] Plasma levels of several cytokines, especially IL-6 and TNF-α, have been described to be increased with age even in apparently healthy individuals and in the absence of acute infection.[12,13] We found that aging has a positive correlation with several markers of vascular inflammation in arteries from menopausal women. Of the 13 markers analyzed, we observed a significant degree

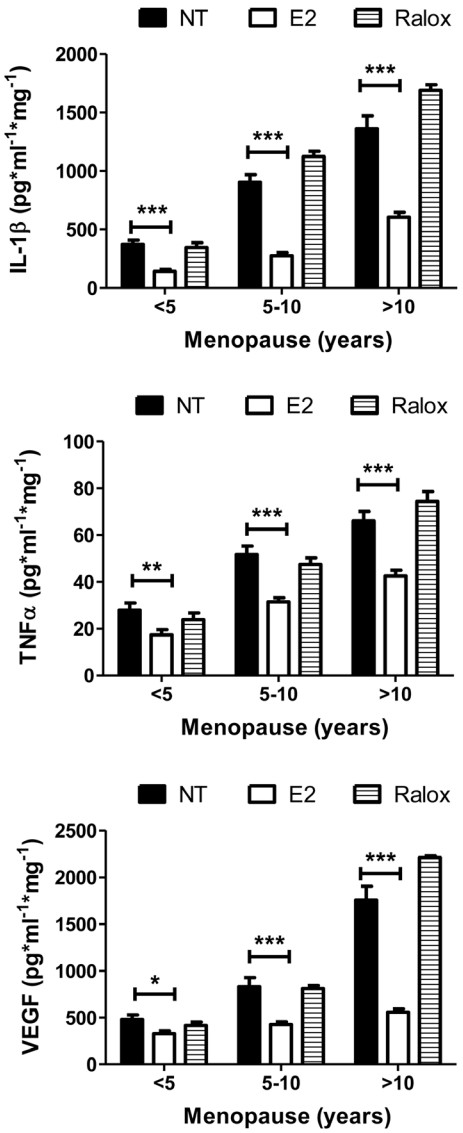


**Figure 2.** Effects of treatments with 17β-estradiol (E2) or raloxifene (ralox) on the expression of inflammatory biomarkers in uterine arteries of women grouped by age, according to time past the onset of menopause: <5 years (<5); between 5 and 10 years (5–10); and >10 years of menopause (>10). Shown are the biomarkers in which E2 displayed an anti-inflammatory action in all age groups. Data are plotted as mean±SEM. **P*<0.05, ***P*<0.01, and *** *P*<0.001. TNF-α indicates tumor necrosis factor-α; VEGF, vascular endothelial growth factor; IL, interleukin.

of correlation in 8 of them, including the adhesion molecules E-selectin, soluble intercellular adhesion molecule-1 and soluble vascular cell adhesion molecule-1, the cytokines IL-1α and TNF-α, and the chemokine monocyte chemotactic protein-1. Our data agree with previous studies establishing that aging process is associated with a development of a more adhesive endothelium. Increased leukocyte adhesion and enhanced expression of adhesion molecules and chemokines have been observed in older individuals compared with young adult,[11] as well as in senescent endothelial cells.[14] On the other hand, even though our results corroborate previous studies showing an aging-associated increase in the cytokines TNF-α and IL-1β, our major surprise was related to the lack of correlation with IL-6, which has been widely described in the literature to be associated with vascular aging process and increased cardiovascular risk. However, the fact that IL-6 concentration was determined in plasma by the majority of studies demonstrates the complexity on the modulation of inflammation in different tissues. Although several inflammatory markers may arise from atheroma plaque, reflecting the extent of inflammation within the vascular lesion, they might also come from nonvascular sources, reflecting inflammatory states, such as chronic infections. As yet, the field still needs to better establish the role and the modulation of the distinct inflammatory markers in the vessel wall during the progression of CVD and aging.

Because women are less likely to progress to CVD, a sex-associated difference in inflammatory responses has been proposed. In fact, recent autopsy studies have described that inflammatory atherosclerosis and associated acute coronary heart disease develop earlier in life in men than in women and are associated with death at an earlier age, although both men and women present the same overall plaque burden.[15] Also in animal models for atherosclerosis, male sex contributes to the progression of lipid deposition, remodeling, and aortic lesions.[16–18] Despite evidences, the mechanisms of such gap are largely unknown, and few studies have addressed this issue by determining the anti- and proinflammatory potential of estrogen in the vasculature. Nonetheless, the available data are rather inconclusive and fairly contradictory. Estrogen has been described to suppress vascular inflammation by down-regulation of proinflammatory molecules, including cytokines and adhesion molecules.[6,7,19–22] Inflammatory response has also been shown to vary significantly according to the estrous cycle in rodents.[23] On the other hand, several clinical studies have described estrogen as a proinflammatory modulator in autoimmune diseases.[5] Meta-analysis of clinical evidence on the modulating effects of estrogens reveals a striking discrepancy with both anti- and proinflammatory effects being described.[4]

There is much controversy over the design and interpretation of the clinical studies to determine the effects of estrogen in the cardiovascular system. Recent studies have suggested that the cardiovascular protective effects of estrogen may be influenced by the type of estrogen and the timing of initiation of estrogen therapy. Furthermore, the most used hormone replacement therapy not only contains estrogenic compounds but also progestins and, importantly, androgens that may interfere with the beneficial effects of estradiol.[24] We have recently shown that equine estrogens display a lower transcriptional activity and lower ability to modulate endothelial function than the naturally occurring estrogen, 17β-estradiol.[25] Consistently, increased inflammatory effects by estrogens were mostly found in women treated with conjugated equine estrogens, whereas others have found reduced or unaffected levels of the same biomarkers when women were treated with 17β-estradiol.[4]

In addition to estrogens, a large number of women are being exposed to selective estrogen receptor modulators to prevent breast cancer recurrence and osteoporosis. According to several experimental and observational studies, the selective estrogen receptor modulator raloxifene has beneficial effects on the vascular system. Similar to estrogen, raloxifene treatment has shown favorable effects on lipid profile and endothelial

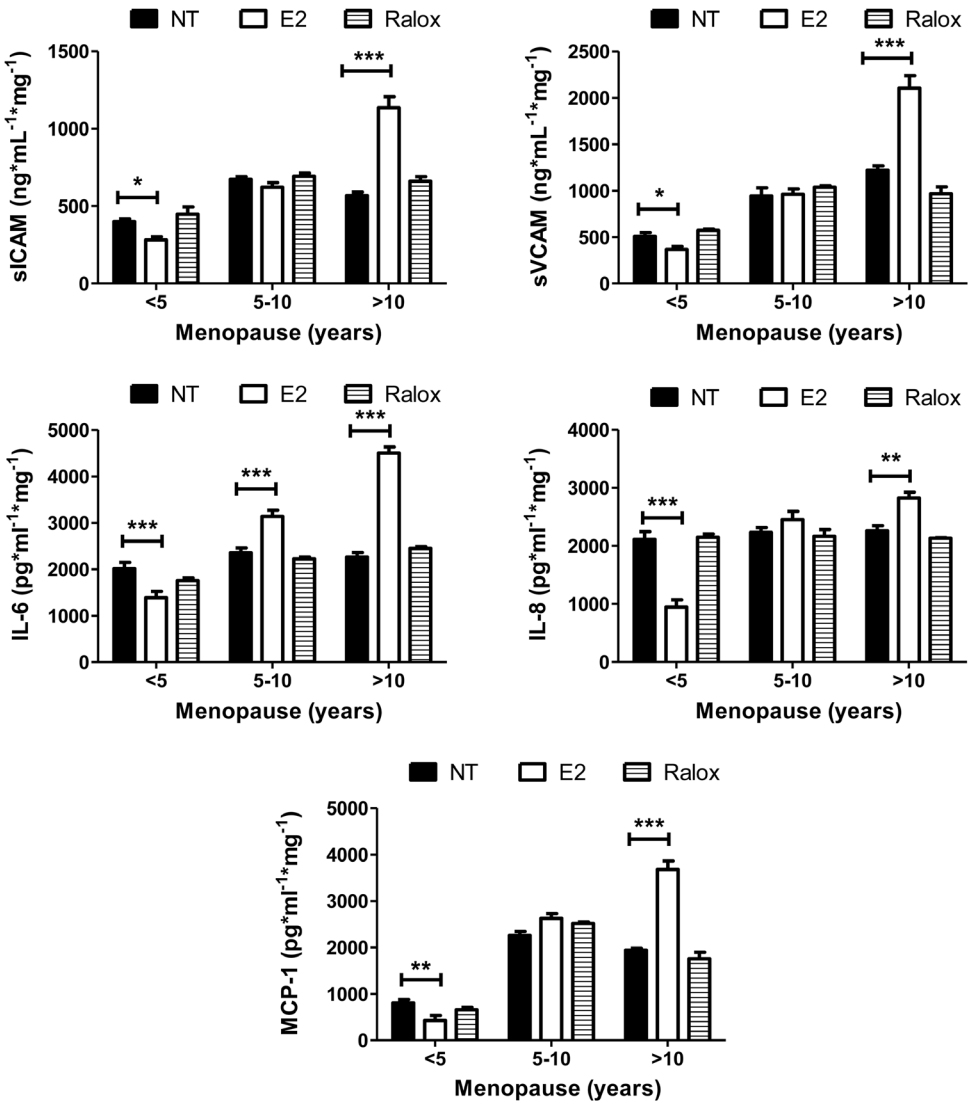


**Figure 3.** Effects of treatments with 17β-estradiol (E2) or raloxifene (ralox) on the expression of inflammatory biomarkers soluble intercellular adhesion molecule-1 (s-ICAM), soluble vascular cell adhesion molecule-1 (s-VCAM), interleukin (IL)-6, IL-8, and monocyte chemotactic protein-1 (MCP1) in uterine arteries of women grouped by age, according to time past the onset of menopause: <5 years (<5); between 5 and 10 years (5–10); and >10 years of menopause (>10). Shown are the biomarkers in which E2 displayed a bidirectional action relative to the time of menopause. Data are plotted as mean±SEM. **P*<0.05, ***P*< 0.01, and ****P*<0.001.

function.[26,27] With regard to its potential as a modulator of vascular inflammation, the little information available reveals similar inconsistency as estrogen.[28] In these studies, we found that short-term treatment with raloxifene does not affect the degree of correlation between time past menopause and biomarkers of inflammation. On the other hand, we observed that 17β-estradiol displays an overall inflammatory effect, because the treatment of arteries with this hormone increases the degree of aging-associated relationship for most biomarkers studied.

However, although our results initially suggest an inflammatory effect by estrogens, a careful look at the data reveals a likely bidirectional effect of estrogen in some inflammatory biomarker that is dependent on age range. In this regard, a second analysis was performed by grouping samples according to time since menopause: up to 5 years of menopause (<5); between 5 and 10 years of menopause (5–10); and >10 years past the onset of menopause (>10). With this analysis, we observed that acute treatment with estrogen induces an anti-inflammatory effect only in arteries from women at early stages of menopause (up to 5 years). In arteries of older women or women who have more years in menopause, estrogen begins to display a timing-dependent proinflammatory effect, suggesting that aging plays a negative role on estrogen-mediated effects on vascular inflammation. In fact, detailed examination of the data from the Women's Health Initiative indicates that early initiation of estrogen replacement produces more favorable results than the late average time of initiation used in the Women's Health Initiative studies overall.[29–31] In support with this hypothesis are few studies showing that aging in female rodents is associated with significant reduction in estrogen-mediated cardiovascular effects.[32–34] In addition, studies have described that timing of onset for estrogen replacement alters the immunologic environment and the progression of vascular inflammation and atherosclerosis formation. Ovariectomized

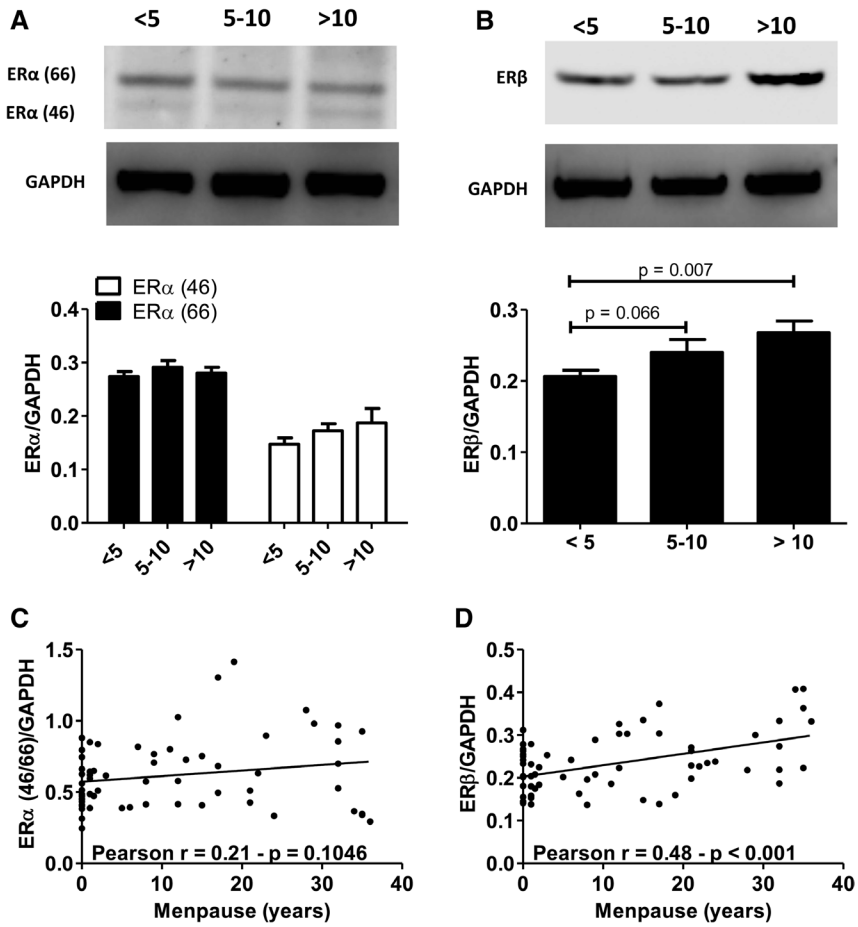


**Figure 4.** Expression of estrogen receptor (ER)-α (**A**) and ER-β (**B**) in untreated uterine arteries of women grouped by age, according to time past the onset of menopause: <5 years (<5); between 5 and 10 years (5–10); and >10 years of menopause (>10). Values for ER-α isoforms, 46 kDa (ER46) and 66 kDa (ER66), and ER-β were normalized by the corresponding optical density for GAPDH, used as the internal control. Data are plotted as mean±SEM. Significant correlation is accepted at $P<0.05$. Simple correlation analysis between time since menopause (*x* axis) and expression of ER-α splicing variants ratio (46kDa/66kDa) (**C**) and ER-β (**D**) (*y* axis) in untreated uterine arteries.

women receiving estrogen immediately after ovariectomy had significantly smaller atherosclerotic lesions than those undergoing delayed or no estrogen exposure.[35]

On the other hand, acute treatment with 17β-estradiol was effective in decreasing the arterial levels of IL-1β and TNF-α in all age groups, despite keeping the positive correlation of those markers with aging. Both are major inflammatory cytokines crucially involved in early stages of vascular inflammation. Through endothelial stimulation, IL-1β and TNF-α increase in synthesis and release of adhesion molecules ICAM and VCAM and set off the inflammatory cascade.[36] Corroborating our data are numerous experimental studies describing that estradiol downregulates TNF-α and IL-1β in different cell types and suggesting an anti-inflammatory and vasculoprotective action by estrogens.[3]

However, although a decrease in levels or action of specific cytokines has been attributed to lower risk of CVD, the functional specificity of each biomarker to the process of chronic inflammation and progression of vascular disease remains uncertain. It is important to note that the mechanisms of actions of these inflammatory markers are highly pleiotropic and often share common pathways and, therefore, could act on other molecules synergistically even when they are at lower concentrations. In fact, there are reports that genetic deletion and pharmacological neutralization of TNF-α fail to reduce atherosclerosis in mice.[37,38] Unfortunately, our study fails to establish how this unbalanced modulation of inflammatory biomarkers (ie, decrease of TNF-α and IL-1β in the presence of augmented levels of other biomarkers, would account for the overall process of vascular inflammation), and even more important is how the acute effects of estrogen observed could be translated into protection or vascular damage in the long run. The major contribution of this study was to demonstrate the aging-associated dichotomy that exists on the effects of estrogen on inflammatory biomarkers, which draws our attention to the need of further prospective studies designed to better establish the effects of estrogen in the process of vascular inflammation and risk of CVD in postmenopausal women.

The mechanisms whereby aging and long-term estrogen withdrawn affect estrogenic responses are largely unknown, but our data suggest a relationship between changes on balance of ERs (ER-α isoforms and ER-β) with increased proinflammatory effect by estrogen. The differences in signaling through ER-α and ER-β are increasingly becoming apparent, and, in fact, previous experimental studies have established that increased expression of ER-β over ER-α is associated with higher oxidative stress and atherosclerotic plaque formation.[39,40] Furthermore, emerging evidences have shown the physiological and pathophysiological relevance of 2 variants of ER-α to estrogen signaling—the full-length ER-α with 66-kDa (ER66) and the ER-α isoform lacking the N-terminal portion (ER46).[41,42] Both isoforms are expressed in the endothelial cells and are able to increase NO production.[41] In this regard, a slight increase in ER46 expression, as observed, would represent a benefit to the vascular wall. However, studies have described that ER46 dimerizes with ER66 and

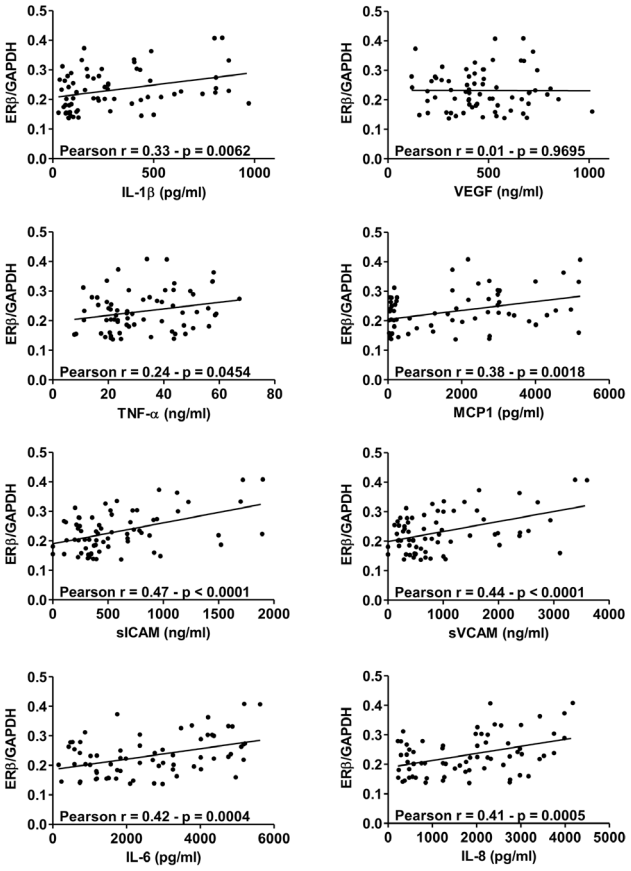


**Figure 5.** Simple correlation analysis between the concentration of biomarkers of inflammation after estrogen treatment (*x* axis) and estrogen receptor (ER)-β expression (*y* axis). Shown are the inflammatory biomarkers that expressed significant increase in the degree of correlation after 17β-estradiol (E2) treatment, as determined by ANCOVA.

serves as a competitive inhibitor of ER66 for DNA binding,[43] and therefore an increase in ER46 may interfere with ER66 transcriptional activity and modify estrogenic responses in a given cell. Such dual effect of ER46 could bias the efficiency of estrogen to modulate vascular inflammation, although we cannot determine in which direction this effect would occur, as both a positive and negative regulation might take place in the vessel wall. Although we interpret the results on changes of ER profile as responsible for reversing the beneficial effects of estrogen, we were unable to provide evidence on whether the relationship between aging and ER expression is worse or modified at long term, or yet, whether it can be altered by estrogen treatment. Nonetheless, the question still arises as to how the crosstalk between ER-α isoforms (ER46 and ER66) and ER-β modulates the effects of estrogen in the vascular wall. Unfortunately, sample size in these studies prevented us from further investigation of these precise mechanisms. Entirely new protocols and new samples are needed to address this issue and to improve our knowledge on the mechanisms for aging and hormonal regulation of vascular function. Despite that, our data demonstrate that estrogen has complex biological effects under distinct pathophysiological conditions and may influence the risk of cardiovascular events and other outcomes. How aging affects estrogen responses and to what extent these changes can modify the risk for CVD remain unknown, but our data strongly suggest that the time to start hormone replacement therapy should be taken into account when deciding the best therapy to treat postmenopausal women.



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## Disclosures

None.

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