Mechanisms Responsible for Endothelial Dysfunction Associated With Acute Estrogen Deprivation in Normotensive Women

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Background—The goal of this study was to evaluate whether endothelial dysfunction associated with acute estrogen deprivation is caused by an alteration in the L-arginine–nitric oxide (NO) pathway and oxidative stress.

Methods and Results—In 26 healthy women (age, 45.7±5.4 years) and 18 fertile women with leiomyoma (age, 44.5±5.1 years), we studied forearm blood flow (strain-gauge plethysmography) changes induced by intrabrachial acetylcholine (0.15, 0.45, 1.5, 4.5, or 15 μg·100 mL⁻¹·min⁻¹) or sodium nitroprusside (1, 2, or 4 μg·100 mL⁻¹·min⁻¹), an endothelium-dependent or -independent vasodilator, respectively. The NO pathway was evaluated by repeating acetylcholine during L-arginine (200 μg·100 mL⁻¹·min⁻¹; 13 control subjects and 9 patients) or N^G-monomethyl-L-arginine (L-NMMA; 100 μg·100 mL⁻¹·min⁻¹; 13 control subjects and 9 patients); production of cyclooxygenase-derived vasoconstrictors was assessed by repeating acetylcholine during indomethacin (50 μg·100 mL⁻¹·min⁻¹; 13 control subjects and 9 patients) or vitamin C (8 mg·100 mL⁻¹·min⁻¹; 13 control subjects and 9 patients). Patients repeated the study within 1 month after ovariectomy and again after 3 months of estrogen replacement therapy (ERT; 17 β-estradiol TTS, 50 μg/d). Basally, vasodilatation to acetylcholine was potentiated and inhibited by L-arginine and L-NMMA, respectively (P<0.05), but was unaffected by indomethacin or vitamin C. After ovariectomy, the modulating effect of L-arginine and L-NMMA disappeared, whereas indomethacin and vitamin C potentiated the response to acetylcholine (P<0.05). ERT restored L-arginine and L-NMMA effects on vasodilation to acetylcholine but prevented the potentiation caused by indomethacin or vitamin C. Response to sodium nitroprusside was unaffected by either ovariectomy or ERT.

Conclusions—Endothelial dysfunction secondary to acute endogenous estrogen deprivation is caused by reduced NO availability. Cyclooxygenase-dependent production of oxidative stress could be responsible for this alteration.

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Key Words: endothelium ■ nitric oxide ■ estrogen ■ antioxidants

Estrogen has a protective action on the vessel wall. Epidemiological studies have demonstrated that menopause and consequent estrogen deprivation increase the risk of cardiovascular disease (CVD) in women.1,2 Moreover, several observational studies have shown a protective effect of estrogen replacement therapy (ERT) against cardiovascular morbidity and mortality in postmenopausal women.3,4 The beneficial effect of estrogen on the cardiovascular system can be partially explained by a positive action on lipid profile.5,6 This hormone also acts directly on the arterial wall by improving endothelial function.

Endothelium plays a primary role in the modulation of vascular tone through production of the relaxing factor nitric oxide (NO), derived from L-arginine by the activity of the enzyme NO synthase,7 which can be specifically inhibited by L-arginine analogs such as N^G-monomethyl-L-arginine (L-NMMA).8 Endothelium can also produce contracting factors that are mainly cyclooxygenase-dependent prostanoids (thromboxane A₂ and prostaglandin H₁)9 or oxygen free radicals, which destroy NO and thus reduce its availability.10 Estrogen has a favorable impact on endothelial function. In normotensive and hypertensive women, endogenous estrogen can prevent or decrease endothelial dysfunction, respectively, associated with increasing age.11,12 In addition, exogenous estrogen can improve endothelium-dependent vasodilation in coronary circulation and peripheral macrocirculation and microcirculation.13-15 However, the mechanism responsible for the beneficial effect of estrogen on endothelial function has not yet been established. To address this issue, we studied normotensive women of reproductive age who underwent...
bilateral ovariectomy and hysterectomy for a uterine leiomyoma. In these patients, the acute estrogen deprivation consequent to ovariectomy is associated with impaired endothelium-dependent vasodilation.16 Our working design was to evaluate whether this endothelial dysfunction secondary to acute estrogen deprivation is characterized by alteration of the L-arginine–NO pathway and production of cyclooxygenase-ase-dependent prostanoids or oxygen-derived free radicals. Finally, the mechanisms through which ERT can restore endothelial dysfunction were also evaluated.

Methods

Subjects

The study population included 18 fertile women 44.5±5.1 years of age (range, 40 to 50 years) with symptomatic uterine leiomyoma associated with menorrhagia (n = 11), pelvic pain (n = 4), and/or rapid increase in size (n = 5) and 26 control healthy women 45.7±5.1 years of age (range, 40 to 52 years) who were well matched for hemodynamic and humoral characteristics (the Table). All patients had regular menstrual cycles. Individuals smoking >5 cigarettes per day and/or consuming >60 g/d ethanol (corresponding to half a liter of wine) were excluded from the study. No patients had received hormone treatment or had a pregnancy for ≥6 months before the study. In accordance with institutional guidelines, the protocol was approved by the local ethics committee. All patients were aware of the investigational nature of the study and gave written consent to it.

Experimental Procedure

Vascular reactivity was assessed by the perfused forearm technique. Briefly, the brachial artery was cannulated for drug infusion at suprasystolic blood pressure. Details concerning the method have already been published.12

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control Subjects (n=26)</th>
<th>Patients (n=18)</th>
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<tbody>
<tr>
<td>Age, y</td>
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<td>44.5±5.1</td>
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<td>Weight, kg</td>
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<td>SBP, mm Hg</td>
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<td>DBP, mm Hg</td>
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<td>Heart rate, bpm</td>
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<td>115.9±7.2</td>
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<td>Plasma glucose, mg/dL</td>
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<td>82.5±6.2</td>
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<td>3/15</td>
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<td>Hemoglobin, g/L</td>
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<td>12.9±1.3</td>
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<tr>
<td>Hematocrit, %</td>
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<tr>
<td>Body mass index, kg/m²</td>
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<td>22.1±1.6</td>
</tr>
<tr>
<td>FBF, mL/100 mL⁻¹·min⁻¹</td>
<td>3.4±0.5</td>
<td>3.4±0.5</td>
</tr>
</tbody>
</table>

OVA indicates ovariectomy; SBP, systolic blood pressure; and DBP, diastolic blood pressure. Values are mean±SD.

Experimental Design

Endothelium-Dependent and Endothelium-Independent Vasodilation

Endothelium-dependent vasodilation was estimated by performing a dose-response curve to intra-arterial acetylcholine (cumulative increase in infusion rates: 0.15, 0.45, 1.5, 4.5, and 15 µg/100 mL forearm tissue per minute for 5 minutes at each dose), whereas a dose-response curve to intra-arterial sodium nitroprusside, a direct smooth muscle cell relaxant compound,18 was performed as control (cumulative increase: 1, 2, and 4 µg/100 mL forearm tissue per minute for 5 minutes at each dose). These rates were selected to induce vasodilation comparable to that obtained with acetylcholine.

Assessment of L-Arginine–NO Pathway

To evaluate the integrity of the L-arginine–NO pathway, we studied the effect of increased NO substrate availability and NO synthase blockade, induced by L-arginine19 and L-NMMA,8 respectively, on endothelial responses. Thus, in 13 of the 26 control subjects and 9 of the 18 patients, the dose-response curve to intra-arterial acetylcholine was repeated in the presence of intrabrachial L-arginine (200 µg/100 mL forearm tissue per minute), whereas in the other subgroups of 13 normal subjects and 9 patients, acetylcholine was repeated during intrabrachial infusion of L-NMMA (100 µg/100 mL forearm tissue per minute). Both L-arginine and L-NMMA were started 10 minutes before acetylcholine and continued throughout.

Assessment of Cyclooxygenase Activity and Oxidative Stress

To evaluate the production of cyclooxygenase-derived factors, we used indomethacin, a cyclooxygenase inhibitor,20 and vitamin C, an antioxidant.21 Thus, in 13 out of the 26 control subjects and 9 of the 18 patients, the dose-response curve to intra-arterial acetylcholine was repeated in the presence of intrabrachial indomethacin (50 µg/100 mL forearm tissue per minute), whereas in the other subgroups of control subjects and patients, the muscarinic agonist was repeated during intrabrachial infusion of vitamin C (8 mg/100 mL forearm tissue per minute). Infusion rates were chosen as the lowest ones producing the maximum effect on acetylcholine in a clinical condition characterized by endothelial dysfunction.22 Indomethacin and vitamin C were started 10 minutes before acetylcholine and continued throughout.

A 30-minute washout was allowed between each dose-response curve. A 60-minute period was allowed when L-NMMA was infused.
In both control subjects and patients, the forearm study was performed during the follicular phase, estimated on the basis of the subject’s previous menstrual cycle. Patients repeated this study within 1 month after ovariectomy (24±2 days) and were subsequently studied again 3 months after ERT (transdermal estradiol; TTS 50, 50 μg/24 h).

As a time-control study, 8 of 26 healthy control women repeated the study after 1 month and subsequently after 4 months. In 1 subgroup of 4 of these 8 control subjects, acetylcholine was infused at baseline and repeated during L-arginine and indomethacin, whereas in the other subgroup, acetylcholine was repeated during L-NMMA and vitamin C administration. The response to sodium nitroprusside was also evaluated. Serum 17β-estradiol concentrations were determined by radioimmunoassay.

**Drugs**

Acetylcholine HCl (Farmigea SpA), indomethacin (Liometacen, Chiesi Farmaceutici SpA), L-arginine and L-NMMA (Clinalfa AG), vitamin C (Bracco), and sodium nitroprusside (Malesci) were obtained from commercially available sources and diluted to the desired concentration by the addition of normal saline. Sodium nitroprusside was dissolved in glucose solution and protected from light by aluminum foil.

**Data Analysis**

Because arterial pressure did not significantly change during the study, all data were analyzed in terms of FBF. FBF increments were taken as evidence of local vasodilation. Study population characteristics shown in the Table were compared by the unpaired Student’s t test. Responses to acetylcholine and sodium nitroprusside were analyzed by ANOVA for repeated measures, and Scheffe’s test was applied for multiple comparison testing. Results were expressed as mean±SD.

**Results**

The hemodynamic and humoral characteristics of the study population are shown in the Table. There was no significant difference in baseline values between control subjects and patients. Moreover, after ovariectomy and ERT, there was no statistically significant modification in basal parameters compared with those obtained before surgery. As expected, plasma estradiol values (basal values, 79.3±22.7 pg/mL) fell to undetectable levels after ovariectomy, subsequently rising to 51.3±16.3 pg/mL after 3 months of ERT.

**Endothelium-Dependent and Endothelium-Independent Vasodilation**

Vasodilation to acetylcholine showed no difference between control subjects (FBF rose from 3.4±0.5 to a maximum of 25.9±4.9 mL/100 mL forearm tissue per minute with the highest dose) and patients before ovariectomy (FBF, from 3.4±0.5 to 25.2±4.8 mL/100 mL·min⁻¹). Similarly, before ovariectomy, the vascular response to sodium nitroprusside was found to be similar both in control subjects (FBF, from 3.5±0.5 to 23.4±3.9 mL/100 mL·min⁻¹) and patients (FBF, from 3.5±0.5 to 22.8±4.1 mL/100 mL·min⁻¹).

After ovariectomy, response to acetylcholine was significantly (P<0.01) blunted (FBF, from 3.4±0.5 to 13.1±3.1 mL/100 mL·min⁻¹) compared with preintervention results. In contrast, the vasodilation to sodium nitroprusside remained unchanged (FBF, from 3.5±0.5 to 23.1±3.7 mL/100 mL·min⁻¹).

Finally, after 3 months of ERT, vasodilation to acetylcholine was significantly (P<0.05) increased compared with values obtained after surgery (FBF, from 3.4±0.4 to 24.7±4.2 mL/100 mL·min⁻¹) and was no longer statistically different from preovariectomy values. In contrast, ERT did not modify the vascular response to sodium nitroprusside (FBF, from 3.4±0.4 to 22.6±4.0 mL/100 mL·min⁻¹).

**Effect of L-Arginine and L-NMMA on Response to Acetylcholine**

At baseline, L-arginine, which did not modify basal FBF (data not shown), significantly (P<0.05) increased the vasodilation to acetylcholine both in healthy women (saline, from 3.4±0.4 to 26.7±4.4 mL/100 mL·min⁻¹; L-arginine, from 3.4±0.6 to 35.6±5.2 mL/100 mL·min⁻¹) and in patients (saline, from 3.5±0.4 to 26.1±3.6 mL/100 mL·min⁻¹; L-arginine, from 3.5±0.4 to 35.1±5.1 mL/100 mL·min⁻¹). This potentiating effect was similar between the 2 groups. After ovariectomy, L-arginine infusion no longer increased vasodilation to acetylcholine (saline, from 3.5±0.4 to 16.1±3.1 mL/100 mL·min⁻¹; L-arginine, from 3.4±0.5 to 16.6±2.8 mL/100 mL·min⁻¹).

Finally, 3 months of ERT restored the potentiating effect of L-arginine on acetylcholine-induced vasodilation (saline, from 3.4±0.3 to 26.5±4.4 mL/100 mL·min⁻¹; L-arginine, from 3.5±0.4 to 35.1±5.1 mL/100 mL·min⁻¹).
34.8±5.6 mL x 100 mL⁻¹ x min⁻¹; P<0.05; Figure 1, top), achieving results comparable to the L-arginine facilitating activity observed before ovariectomy.

In healthy women, L-NMMA, which caused a decrease in basal FBF (from 3.3±0.5 to 2.0±0.5 mL x 100 mL⁻¹ x min⁻¹; P<0.01), significantly blunted the vasodilating effect of acetylcholine (saline, from 3.5±0.6 to 25.4±4.2 mL x 100 mL⁻¹ x min⁻¹; L-NMMA, from 2.0±0.5 to 7.2±1.3 mL x 100 mL⁻¹ x min⁻¹; P<0.05 versus acetylcholine alone). Likewise, in patients, L-NMMA decreased basal FBF (from 3.1±0.2 to 2.0±0.2 mL x 100 mL⁻¹ x min⁻¹; P<0.05) similar to that obtained in normal control subjects and blunted the vasodilation to acetylcholine (saline, from 3.2±0.4 to 24.3±3.1 mL x 100 mL⁻¹ x min⁻¹; L-NMMA, from 2.1±0.2 to 7.2±1.9 mL x 100 mL⁻¹ x min⁻¹; Figure 1, bottom), whereas ERT restored the blunting effect of the NO synthase inhibitor on vasodilation to acetylcholine (saline, FBF from 2.0±0.4 to 27.7±2.1 mL x 100 mL⁻¹ x min⁻¹; Figure 1, bottom). Values were thus no longer statistically different from preovariectomy levels.

Effect of Indomethacin and Vitamin C on Response to Acetylcholine

At baseline, indomethacin and vitamin C did not modify basal FBF (data not shown) or vasodilation to acetylcholine in either healthy women (saline, from 3.4±0.4 to 26.7±4.4 mL x 100 mL⁻¹ x min⁻¹; indomethacin, from 3.4±0.4 to 25.6±4.0 mL x 100 mL⁻¹ x min⁻¹; and saline, from 3.5±0.6 to 25.4±4.2 mL x 100 mL⁻¹ x min⁻¹; vitamin C, from 3.5±0.6 to 24.4±4.2 mL x 100 mL⁻¹ x min⁻¹) or patients (saline, from 3.5±0.4 to 26.1±3.6 mL x 100 mL⁻¹ x min⁻¹; indomethacin, from 3.4±0.5 to 24.3±3.9 mL x 100 mL⁻¹ x min⁻¹; and saline, from 3.2±0.4 to 24.3±3.1 mL x 100 mL⁻¹ x min⁻¹; vitamin C, from 3.2±0.4 to 23.5±3.4 mL x 100 mL⁻¹ x min⁻¹; Figure 2, bottom).

After ovariectomy, indomethacin and vitamin C infusion still had no effect on basal FBF but significantly (P<0.05) increased vasodilation to acetylcholine (saline, from 3.4±0.4 to 15.1±3.3 mL x 100 mL⁻¹ x min⁻¹; indomethacin, from 3.3±0.4 to 22.4±3.6 mL x 100 mL⁻¹ x min⁻¹; and saline, from 3.2±0.4 to 13.0±2.9 mL x 100 mL⁻¹ x min⁻¹; vitamin C, from 3.3±0.4 to 22.1±3.8 mL x 100 mL⁻¹ x min⁻¹; Figure 2, bottom). The potentiating effect on the response to acetylcholine exerted by indomethacin and vitamin C was similar (163% and 154% increase in the area under the curve of the dose response to acetylcholine, respectively). Finally, after 3 months of ERT, indomethacin and vitamin C were again found to be ineffective on acetylcholine-induced vasodilation (saline, from 3.4±0.5 to 23.9±4.0 mL x 100 mL⁻¹ x min⁻¹; indomethacin, from 3.4±0.5 to 23.4±4.3 mL x 100 mL⁻¹ x min⁻¹; and saline, from 3.3±0.4 to 23.6±4.4 mL x 100 mL⁻¹ x min⁻¹; vitamin C, from 3.2±0.6 to 22.9±4.0 mL x 100 mL⁻¹ x min⁻¹; Figure 2, bottom). In both control subjects and patients, contralateral FBF showed no significant change throughout the study (data not shown).

Time-Control Study

In these subjects, the response to acetylcholine was increased and decreased by L-arginine and L-NMMA, respectively, whereas both indomethacin and vitamin C were ineffective (Figure 3). These responses were found to be similar when repeated 1 and 4 months after the baseline study (Figure 3). Vasodilation to sodium nitroprusside was also unchanged (data not shown).

Discussion

The present study shows that acute endogenous estrogen deprivation after ovariectomy impairs vasodilation to acetylcholine but not to sodium nitroprusside, a negative effect that is reversed by ERT administration. Taken together, these results confirm previous evidence indicating that estrogen has a protective effect on endothelium-dependent vasodilation.²⁶ However, the original finding of this study concerns the mechanisms responsible for endothelial dysfunction induced by acute endogenous estrogen deprivation in women. At baseline, we observed that in healthy control women the
vasodilation induced by acetylcholine was potentiated and inhibited by L-arginine and L-NMMA, respectively. Similar results were observed in the leiomyoma patients before surgical intervention, indicating a preserved L-arginine–NO pathway. In contrast to baseline, after ovariectomy, L-arginine no longer improved vasodilation to acetylcholine, and L-NMMA failed to blunt the response to the muscarinic agonist, demonstrating that acute endogenous estrogen deprivation leads to an alteration in agonist-induced NO pathway activation. This possibility seems to be confirmed by the finding that ERT restores the facilitating and inhibiting effect of L-arginine and L-NMMA, respectively.

Concerning the effect of L-arginine on acetylcholine-induced vasodilation, conflicting results are available in literature; some authors demonstrate a potentiating effect, and others demonstrate no effect. This discrepancy could be explained by taking into account the age of the healthy study population. In young subjects, L-arginine seems to be devoid of effect on the response to acetylcholine, but in older individuals, L-arginine also increase endothelial NO synthase expression, thereby augmenting NO production.

It is worth noting that baseline FBF and the vasoconstricting effect of L-NMMA underwent no change during the study, indicating that NO-dependent regulation of basal vascular tone is not affected by ovariectomy. This result is in line with previous evidence indicating that agonist-induced NO availability is modulated by mechanisms different from basal NO production.

Concerning the cyclooxygenase pathway, at baseline in both healthy women and patients with leiomyoma, indomethacin had no effect on vasodilation to acetylcholine, as already demonstrated. In contrast, after ovariectomy, indomethacin significantly potentiated vasodilation to acetylcholine, indicating the appearance of cyclooxygenase-dependent factors as a mechanism contributing to this endothelial alteration. Thus, estrogen deprivation secondary to ovariectomy leads to activation of the cyclooxygenase pathway, which seems to participate in the impaired endothelium-dependent vasodilation. This interpretation is confirmed by results obtained after ERT administration, which prevents the potentiation caused by indomethacin. No available experimental data support the possibility that cyclooxygenase-derived factors lead to endothelial dysfunction under estrogen deprivation. However, in essential hypertension, a clinical condition characterized by endothelial dysfunction, cyclooxygenase seems to be a source of oxidative stress.

Similar results have been obtained with vitamin C. In basal conditions in both healthy control women and leiomyoma patients, we observed that vitamin C did not affect the vascular response to acetylcholine, suggesting that oxygen free radical production does not occur. In contrast, after ovariectomy, vitamin C significantly potentiated endothelium-dependent vasodilation, suggesting a possible role of oxygen free radicals in determining endothelial dysfunction after acute estrogen deprivation. This hypothesis is confirmed by evidence that after ERT the antioxidant no longer had an effect on vasodilation to acetylcholine. It is worth noting that after ovariectomy vitamin C caused a potentiating effect on the response to acetylcholine of the same degree as that exerted by indomethacin, again suggesting that in essential hypertension as well, the cyclooxygenase pathway could be a source of oxygen free radical. It is important to point out, however, that in our experimental condition we can only speculate that vitamin C acts as a scavenger for oxygen free radicals, because we cannot exclude a different mechanism, such as interference with the pathway responsible for oxygen free radical production.

The possible antioxidant effect of estrogen is in agreement with the experimental finding that the hormone can preserve endothelium-dependent vasodilation by preventing oxygen free radical production. However, the lack of substantial experimental evidence, together with the unfavorable chemistry of vitamin C as an oxygen free-radical scavenger, casts considerable doubt on the possible role of oxidative stress as the cause of endothelial dysfunction after acute estrogen deprivation. On the other hand, it must be taken into account that estrogen can also increase endothelial NO synthase expression, thereby augmenting NO production. In line with this reasoning, Rosselli et al. demonstrated that in postmenopausal women long-term estrogen replacement can increase serum nitrate and nitrite levels, an index of enhanced NO production. In addition, the potentiating effect on endothelial function of acute estrogen administration is inhibited by L-NMMA.

As regards the clinical relevance of the present results, it is important to observe that endothelial dysfunction is associated with several cardiovascular risk factors, such as aging, essential hypertension, postmenopause, diabetes mellitus, hypercholester-
olemia, smoking, and hyperhomocysteinemia. Because general agreement exists that endothelial dysfunction could be an early promoter of atherosclerosis in patients with cardiovascular risk factors, it is conceivable that endothelial dysfunction secondary to estrogen deficiency could be at least partially responsible for the increased risk of CVD in postmenopausal women. Therefore, the beneficial impact of exogenous estrogen administration on CVD in this population could be at least partially mediated by an improvement in endothelial function. However, concomitant progestin administration could represent a possible limitation on the beneficial effect of exogenous estrogen on endothelial function. At the present time, 3 studies are available in humans, with conflicting results. Therefore, it remains to be validated whether concomitant administration of progestin can oppose the effect of estrogen on endothelial function. This represents a crucial issue, considering the possible use of ERT in primary and secondary cardiovascular prevention. In this regard, a recent placebo-controlled trial, the Heart and Estrogen/Progestin Replacement Study, showed that estrogen plus progestin therapy did not reduce the rate of coronary events in postmenopausal women with established coronary disease. Finally, the Women’s Health Initiative will provide data concerning the possible effect of ERT on primary prevention of cardiovascular events in postmenopausal women.

References

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