Part I

Transdermal hormone therapy
Oral, more than transdermal, estrogen therapy improves lipids and lipoprotein(a) in postmenopausal women: a randomized, placebo-controlled study

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Abstract

**Objective:** To assess the effects of low dose oral and transdermal estrogen therapy on the lipid profile and lipoprotein(a) [Lp(a)] levels in healthy postmenopausal women and to study the additional influence of gestodene administration.

**Design:** In a multicenter, randomized, double-blind, placebo-controlled study, 152 healthy, hysterectomized, postmenopausal women received daily either placebo (n=49), 50 μg transdermal 17β-estradiol (tE2, n=33), 1 mg oral 17β-estradiol (oE2, n=37), or 1 mg oE2 combined with 25 μg gestodene (oE2+G, n=33) for 13 cycles of 28 days, followed by 4 cycles of placebo in each group. Fasting serum concentrations of total, high-density lipoprotein (HDL) cholesterol and low-density lipoprotein (LDL) cholesterol, triglycerides and Lp(a) were measured at baseline and in cycles 4, 13 and 17.

**Results:** In cycle 13, a significant mean percentage decrease from baseline was found in all treatment groups compared with placebo in total cholesterol (tE2 -4.7%; oE2 -6.9%; oE2+G -10.5%) and LDL cholesterol (tE2 -5.8%; oE2 -12.6%; oE2+G -13.6%). For both oral groups, the reductions were already significant in cycle 4. None of the treatment groups showed a significant change in HDL cholesterol or triglycerides. In cycle 13, Lp(a) was decreased compared to placebo in the oE2 group (-6.6%) and the oE2+G group (-8.2%). After washout, all observed changes had returned to baseline level, except for the decreases in total and LDL-cholesterol in the oE2+G group.

**Conclusions:** Oral E2 and E2+G, and to a lesser extent transdermal E2, decreased total and LDL cholesterol. Lp(a) was lowered only by the oral treatments.
Introduction

Cardiovascular disease is the leading cause of death among postmenopausal women. Whereas before menopause cardiovascular disease is uncommon in women, after menopause the incidence increases towards a pattern comparable to that seen in males.\(^1\) This has been attributed to a modification of cardiovascular risk factors that accompanies the menopausal transition. Lipids and lipoproteins, which are important determinants of this risk, undergo an unfavorable change after menopause.\(^2\) Compared with premenopausal levels, an increase in total cholesterol, low-density lipoprotein (LDL) cholesterol and triglycerides have been reported, together with a decrease in high-density lipoprotein (HDL) cholesterol.

Lipoprotein(a) \([\text{Lp(a)}]\) is a highly atherogenic particle consisting of apo(a), which is linked to a complex of LDL and apoB-100 with a disulfide-link. A high serum level of Lp(a) is another independent risk factor for the development of atherosclerosis and thrombosis.\(^3,4\) Menopause is associated with an increase in Lp(a) levels.\(^5\) Lipid-lowering drugs have shown to reduce cardiovascular morbidity by lowering the lipid levels,\(^6\) but only niacin has been shown to also reduce Lp(a) levels.\(^7\)

Observational studies have demonstrated a decrease in cardiovascular events among users of postmenopausal estrogen therapy (ET).\(^8,9\) This effect has been attributed to a combined effect on several risk factors, part of which is accounted for by improving the lipid levels.\(^10\) In contrast to statins, ET has also been reported to induce a reduction in Lp(a) levels.\(^11\) In the last few years, the results of the first randomized, placebo-controlled studies on the effects of hormone therapy (HT) on cardiovascular events have been published.\(^12-14\) These showed a beneficial effect of ET and HT on lipids and lipoproteins.\(^12,13\) However, these studies did not find a reduction in clinical outcome measures, but even an early higher risk for coronary heart disease (CHD) and an increase in venous thromboembolism were seen. Pro-thrombotic effects of oral ET\(^15\) may well explain this risk.

For systemic estrogen treatment, several routes of administration are currently available: oral, transdermal, subcutaneous and intranasal. These routes may elicit different effects on cardiovascular risk parameters.

In general, the effects of oral ET are beneficial in that they lower total cholesterol, LDL cholesterol and Lp(a), and increase HDL-cholesterol levels.\(^10\) However, an unfavorable effect is an increase in triglyceride levels. Compared with conjugated equine estrogens, 17β-estradiol (E\(_2\)) induces a smaller effect on HDL cholesterol and triglyceride levels.\(^10\) Lowering the dose also diminishes the effect on HDL cholesterol and triglycerides levels.\(^10\) Transdermal estrogen, the most widely studied non-oral administration route, has been shown to induce similar but smaller effects on total, LDL and HDL cholesterol, but also to induce a beneficial decrease in triglyceride levels.\(^10\) Addition of a progestogen has been reported to attenuate the estrogen-induced changes. The impact on the changes is related to the type and dosage of the progestogen used as well as the route and duration of administration. Progestogens especially can modify the estrogen-induced effect on HDL cholesterol and triglycerides.\(^10\)

Gestodene is a third generation progestogen, the effects of which have mainly been described for the use in oral contraceptives containing ethinyl estradiol.\(^16\) Recently, a beneficial effect on the lipid profile of gestodene used in combination with estradiol has been described.\(^17,18\)

High levels of Lp(a) can beneficially be influenced by oral HT.\(^10,11\) A few studies are available on transdermal ET\(^19-22\) and Lp(a), but none on the effect of gestodene. In the present randomized,
placebo-controlled trial, we compared the effects of transdermal and oral estrogen therapy on Lp(a) and on conventional lipids. Furthermore, the additional influence of oral gestodene on estradiol-induced changes was studied.

Methods

Participants

Healthy, postmenopausal women who had undergone a hysterectomy (not necessarily combined with an oophorectomy) were recruited through advertisements in the local newspapers between May 1997 and February 1999. The last follow-up visit was in May 2000. After screening, 152 women were eligible and enrolled in this study, which was performed at the outpatient clinics of the Departments of Obstetrics and Gynecology at four hospitals in the Netherlands (in Amsterdam, Nijmegen, Utrecht, and Enschede). The investigation conformed to the principles outlined in the Declaration of Helsinki. All Institutional Review Boards approved the protocol. Written informed consent was obtained from each participant before entry into the study.

All participants were between 45 and 65 years of age, were nonsmokers or smoked fewer than six cigarettes per day, had blood pressures below 160 mmHg systolic and 100 mmHg diastolic, and had a body mass index (BMI) of 30 kg/m² or less. Postmenopausal status was defined as having serum follicle-stimulating hormone (FSH) concentrations greater than 40 IU/L and estradiol concentrations lower than 110 pmol/L on each of two different visits during the screening period. None of the women had received HT within 6 months before randomization, and none took cardiovascular medication, lipid-lowering drugs, or drugs known to influence estrogen or progestogen metabolism. Exclusion criteria included a history of cardiovascular, venous thromboembolic, metabolic, endocrinological (except for thyroid disease in a stable phase), and malignant disease (except for successfully resected basal skin cancer), as well as clinically relevant abnormalities in laboratory tests of hematological, renal and hepatic function, or glucose metabolism. Women with fasting plasma cholesterol levels above 8 mmol/L were excluded as well.

To prevent participants, clinical investigators, and laboratory personnel from becoming aware of the medication used, we employed a double-blind, double-dummy approach. This approach may also have helped keep women randomized to placebo from starting other active treatment to relieve symptoms. Eligible women were randomly assigned to either a placebo tablet and placebo patch (placebo group; n=49); to transdermal 17β-estradiol (50 μg daily, Climara®) and a placebo tablet (tE₂ group, n=33); to oral micronized 17β-estradiol (1 mg daily) and a placebo patch (oE₂ group, n=37); or to one tablet daily of oral micronized 17β-estradiol (1 mg) plus gestodene (25 μg) and a placebo patch (oE₂+G group, n=33), given for the first 13 28-day cycles, followed by four cycles of placebo tablets and placebo patches for each group. Tablets were taken daily, and patches were changed once every seven days. Medication was manufactured, packaged and labeled by Schering AG, SBU Fertility Control & Hormone Therapy (Berlin, Germany). All tablets were identical in appearance and organoleptic characteristics, as were all patches. Computerized randomization was done in blocks, which had a size of 13 and consisted of four times placebo treatment and three times each active treatment for the first thirteen cycles plus placebo treatment for the last four cycles. We included more women in the placebo group than in the other groups, as we expected more dropouts in this
group. The allocation sequence was generated by the statistical department of Schering AG. Enrollment of participants and assignment to the treatment was done by the investigator at each hospital. Medication boxes were numbered and allocation was done in sequence. The blinding was discontinued after lipids and Lp(a) concentrations were measured at the end of the study.

A total of 363 women were screened, of whom 152 participants were enrolled. The main reason for screen-failure (n=211) was a participant not being postmenopausal (n=153). Other reasons were abnormal laboratory findings at screening (n=20), medical history or use of concomitant medication (n=11), abnormal findings at physical examination (n=10) and withdrawal of consent (n=17). The different centers included 66, 37, 33 and 16 women, respectively. From one woman of the oE2+G group, it was not possible to obtain sufficient blood samples during the study. Three women dropped out before the measurements in cycle 4: two women in the placebo group because of withdrawal of consent and one woman in the tE2 group because of breast tenderness and edema. So, in cycle 4, blood samples were collected from 148 women. Reasons for dropout between the measurement in cycle 4 and in cycle 13 (total n=7) were, in the placebo group: skin reaction to the patches (n=1), ovarian carcinoma (n=1) and appendicitis/peritonitis (n=1); in the tE2 group: breast tenderness (n=1) and skin reaction to the patches (n=1); in the oE2 group: bowel complaints (n=1); and in the oE2+G group: withdrawal of consent (n=1). In addition, in one woman in the oE2+G group it was not possible to obtain sufficient blood samples in cycles 13 and 17. So, in cycle 13, blood samples were collected from 140 women. After the measurements in cycle 13, when all women received placebo, another 6 women dropped out. The reasons were, in the placebo group: osteoporosis (n=1), climacteric complaints (n=2), and withdrawal of consent (n=1); in the tE2 group: depressive feelings (n=1); and in the oE2+G group: climacteric complaints (n=1). In another two women, one in the oE2 group and one in the oE2+G group, insufficient blood samples were obtained for the lipid measurements, excluding Lp(a). Therefore, the analysis of the washout period for women completing the trial was based on 132 women for lipids and 134 women for Lp(a) (Figure 1).

Lipids and Lipoprotein(a) measurements

At baseline and in cycle 4 (cycle day 1 to 14), cycle 13 (cycle day 1 to 28) and cycle 17 (cycle day 1 to 28) of follow-up, venous blood samples were taken between 8.00 a.m. and 10.00 a.m., with participants in a supine position. The participant had fasted and refrained from smoking for at least 10 hours and had not consumed alcohol for at least 24 hours. After 20 minutes of rest, blood was collected with a Vacutainer® system (Becton Dickinson, Meyren, Cedex-France) into separate plain tubes (Becton Dickinson, Plymouth, UK). Serum was separated by centrifugation at 3,000 g at 20°C for 10 minutes within 1 hour of collection. Serum was divided into aliquots, snap-frozen and stored at −80°C until analysis.

Serum lipid levels were measured with automated assays on a Hitachi 747 analyzer (Roche, Mannheim, Germany). For total cholesterol, HDL cholesterol, and triglycerides, the following reagents were used: CHOD–PAP, HDL-C plus, and GPO-PAP, respectively (all by Roche, Mannheim, Germany). The intra-assay coefficients of variation (CV) were 0.61%, 1.80%, and 0.62%, respectively, the interassay CV were 2.1%, 3.3%, and 5.1%, respectively. LDL cholesterol was calculated using the Friedewald-formula.25 Serum Lp(a) concentrations were determined using a commercially available enzyme-linked immunoassay (Innotest, Innogenetics NV, Zwijndrecht, Belgium). The intra-assay CV for this ELISA was 3.6%, and the interassay was CV 4.5%. All samples for a given subject were assayed within a single run.
Other measurements

Serum FSH was determined with a specific immunometrical (luminescence) assay (Amerlite, Amersham, Little Chalfont, UK). Serum estradiol was quantified by using a double-antibody radioimmunoassay (Sorin Biomedica, Saluggia, Italy) with a lower limit of detection of 18 pmol/L.

Statistics

Statistical analysis was performed using the Statistical Package for the Social Sciences PC + 4.0 (SPSS Inc., Chicago, IL, USA). Values are given as mean ± standard deviation for cholesterol, or as median (range) for triglycerides and Lp(a). Analyses of triglycerides and Lp(a) were performed after log transformation. We compared measurements between groups using standard parametric tests; only for descriptive characteristics were nonparametric tests used where applicable. Correlations at baseline were calculated with Spearman’s correlation coefficient. Analyses of covariance (ANCOVA) for repeated measurements with the baseline value as covariate, were used for comparisons among and between the groups.

In the ANCOVA, results from 148 women, for whom data were available at baseline and cycle 4, were analyzed using the last-observation-carried-forward procedure for the missing values of 8 women at cycle 13.
Sample size calculation was based on the primary outcome variable, HDL cholesterol. To detect a mean difference of 0.2 mmol/L among the treatment groups and the placebo group with 80% power using a two-sided test at the 5% significance level, 36 evaluable women in each treatment arm were required.

Results

The groups were comparable with respect to age, BMI, blood pressure, smoking habits, total cholesterol, serum FSH and E2 levels (Table I) and alcohol consumption (data not shown).

### Table I. Descriptive characteristics of the four groups at baseline

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>tE2</th>
<th>oE2</th>
<th>oE2+G</th>
<th>P-value</th>
</tr>
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<tbody>
<tr>
<td>n</td>
<td>49</td>
<td>33</td>
<td>37</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>55.0 ± 4.7</td>
<td>55.5 ± 4.8</td>
<td>54.4 ± 4.3</td>
<td>53.4 ± 4.2</td>
<td>0.24</td>
</tr>
<tr>
<td>Body mass index (kg/m2)</td>
<td>25.7 ± 3.0</td>
<td>26.0 ± 2.4</td>
<td>25.0 ± 3.2</td>
<td>26.2 ± 3.1</td>
<td>0.30</td>
</tr>
<tr>
<td>Blood pressure</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>systolic (mmHg)</td>
<td>128 ± 17</td>
<td>134 ± 14</td>
<td>127 ± 12</td>
<td>126 ± 17</td>
<td>0.14</td>
</tr>
<tr>
<td>diastolic (mmHg)</td>
<td>79 ± 8</td>
<td>83 ± 9</td>
<td>81 ± 10</td>
<td>79 ± 9</td>
<td>0.23</td>
</tr>
<tr>
<td>Smokers n (%)</td>
<td>7 (14)</td>
<td>1 (3)</td>
<td>3 (8)</td>
<td>4 (12)</td>
<td>0.37</td>
</tr>
<tr>
<td>Serum cholesterol (mmol/L)</td>
<td>5.9 ± 0.9</td>
<td>6.1 ± 0.9</td>
<td>6.2 ± 1.0</td>
<td>5.9 ± 1.1</td>
<td>0.39</td>
</tr>
<tr>
<td>Serum FSH (U/L)</td>
<td>62 ± 20</td>
<td>63 ± 23</td>
<td>67 ± 28</td>
<td>67 ± 32</td>
<td>0.72</td>
</tr>
<tr>
<td>Serum E2 (pmol/L)</td>
<td>29 (18; 402)</td>
<td>28 (18; 912)</td>
<td>29 (18; 192)</td>
<td>29 (18; 333)</td>
<td>0.98</td>
</tr>
</tbody>
</table>

Values are given as mean ± SD, median (range) or as number (n) with percentage in parentheses. tE2, transdermal 17β-estradiol 50 μg; oE2, oral 17β-estradiol 1 mg; oE2+G, oE2 plus gestodene 25 μg; FSH, follicle-stimulating hormone; E2, estradiol.

aOne-way ANOVA or Kruskal-Wallis- or χ2-test for between-group differences.

Lipids

One-way ANOVA did not show significant differences among the treatment groups for baseline values.

With the baseline value as covariate, ANCOVA over the first 13 cycles showed an overall difference among groups in total cholesterol (P<0.001), LDL cholesterol (P<0.001), and triglycerides (P=0.039) (Table II). A significant difference was found for active treatment versus placebo (total cholesterol, P<0.001; LDL cholesterol, P<0.001), and for oral versus transdermal treatment (total cholesterol, P=0.007; LDL-cholesterol, P=0.002). For HDL cholesterol and triglycerides, these comparisons were not significant.

In cycle 13, the mean percentage decrease from baseline in total cholesterol compared with placebo was significant in all active treatment groups (tE2 -4.7%, P=0.03; oE2 -6.9%, P=0.002; oE2+G -10.5%, P<0.001). For both oral treatment groups, this difference was already significant in the fourth cycle (oE2 -5.2%, P=0.02; oE2+G -9.2%, P<0.001) (Figure 2).
A significant percentage decrease from baseline was also found for LDL cholesterol in cycle 13 in all active treatment groups compared with placebo (tE2 -5.8%, P=0.04; oE2 -12.6%, P<0.001; oE2+G -13.6%, P<0.001). The difference was already significant in the fourth cycle for both oral treatment groups (oE2 -10.2%, P<0.001; oE2+G -11.2%, P<0.001).

A significant percentage change compared with placebo in HDL cholesterol and triglycerides was not found in any of the active treatment groups after 13 cycles of treatment. But in cycle 4, the percentage change in HDL cholesterol differed significantly between the oE2 (+3.9%) and the tE2 group (-1.4%) as well as the oE2+G group (-2.9%). The change in triglycerides in cycle 13 in the oE2 group (+12.5%) differed significantly from the tE2 group (0%) as well as the oE2+G group (-6.9%); the difference between both oral treatment groups was already significant in cycle 4.
Figure 2. Percentage changes in lipid profile

Percentage changes versus baseline. Values given as mean or median.
*P<0.05; **P<0.01; ***P<0.001; @P=0.10 for the comparison between active treatment and placebo.
#P<0.05 and ##P<0.01 for the comparison between two active treatment groups.

**Transdermal hormone therapy**

- **a. Total cholesterol**
  - Placebo
  - tE2 (transdermal 17β-estradiol)
  - oE2 (oral 17β-estradiol)
  - oE2+G (oral 17β-estradiol + gestodene)

- **b. HDL-cholesterol**

- **c. LDL-cholesterol**

- **d. Triglycerides**

- **e. Lipoprotein(a)**

Lipids and Lipoprotein(a)
Lipoprotein(a)

At baseline, levels of Lp(a) correlated positively with total cholesterol \((r=0.17, P=0.04)\) and LDL cholesterol \((r=0.23, P=0.004)\). One-way ANOVA for cross-sectional comparison of the baseline values of Lp(a) among the treatment groups showed a difference, which reached statistical significance \((P=0.04)\). The values of the oE\(_2\) \((P=0.01)\) and oE\(_2\)+G group \((P=0.053)\) differed from placebo.

With the baseline value of Lp(a) as covariate, ANCOVA over 13 cycles showed a change in Lp(a) among the groups \((\text{overall } P=0.01)\). Comparison of all active treatment groups with placebo just failed to reach statistical significance \((P=0.066)\). However, the reductions observed with oral treatment were significantly larger than with placebo \((P=0.006)\) or transdermal \((P=0.004)\) (Table III).

In the 13th cycle a median percentage decrease from baseline compared with placebo was found for the oral treatment groups \((\text{oE}_2 \text{-6.6%, } P=0.10; \text{oE}_2+\text{G -8.2%, } P=0.03)\) but not for the transdermal group \((\text{tE}_2 +7.0%, P=0.66)\) (Figure 2).

In the oE\(_2\)+G group, the decrease was more in women with baseline values in the highest than the lowest tertile \((P<0.001)\). For the other treatment groups no significant differences were found.

<table>
<thead>
<tr>
<th>Table III. Lp(a) concentrations</th>
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<tr>
<td></td>
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<tr>
<td>Lp(a)</td>
</tr>
<tr>
<td>tE(_2)</td>
</tr>
<tr>
<td>oE(_2)</td>
</tr>
<tr>
<td>oE(_2)+G</td>
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</table>

Values at baseline, cycle 4, 13 and 17 are concentrations (in mmol/L) as median (range).
\(a\)Percentage change from baseline expressed as median (range).
\(b\)Analysis of covariance for repeated measurements with the baseline value as covariate over the 13-cycle period (after log transformation): treatment group versus placebo; \(^c\)overall.

P-values: t-test for between-group differences compared to placebo: \(^dP < 0.05; ^eP < 0.01.\)

Washout

In the fourth washout cycle (cycle 17), all changes observed in the 13th cycle had returned towards the baseline value. A significant percentage decrease from baseline compared with placebo persisted for total cholesterol \((-5.9%, P=0.04)\) and LDL cholesterol \((-9.1%, P=0.03)\) only in the oE\(_2\)+G group.

Discussion

In the present study, we found a beneficial effect of three different regimens of 17β-estradiol on lipid and lipoprotein levels. Oral estradiol, unopposed as well as combined with gestodene, and to a lesser extent transdermal estradiol, were able to induce a reduction in both total and LDL cholesterol. Oral but not transdermal treatment significantly reduced Lp(a) levels. These findings are consistent with earlier studies, many of them with higher doses of estradiol.
Because it avoids the hepatic first-pass effect seen after oral administration, transdermal ET induces metabolic changes that differ from those observed with oral ET. Overall, studies on transdermal unopposed estradiol, most involving patches releasing 50 μg estradiol daily, showed a decrease in total and LDL cholesterol and an increase in HDL cholesterol and, in contrast to oral estrogens, a decrease in triglycerides. The changes are smaller than induced by oral estrogens and often miss significance. We found no decrease in triglyceride levels, although the effect differed significantly from the increase observed in the oral estradiol group.

Several studies have described the effect of combined transdermal HT on Lp(a) levels, but only a few reported on unopposed transdermal estradiol on Lp(a). As in our study, none found significant changes in Lp(a) levels. Where these studies assessed the effect within 6 months, in our study still no effect was seen after one year of treatment.

The effect on lipids of a progestogen added to ET depends on the type of progestogen used. The estrogen-induced increase in HDL cholesterol and triglycerides is attenuated by progestogens, or even totally neutralized. The decrease in Lp(a) observed during continuous-combined hormone treatment is equal to or even greater than with unopposed estrogen. Studies that compared the effect of combined treatment with unopposed CEE or estradiol are sparse.

Gestodene is a 19-nortestosterone progestogen that is mainly used in oral contraceptives. Its effect on lipids when used in combination with estradiol has been described in two studies. In both studies, the combined oral estradiol (1 mg) and gestodene regimens induced a significant decrease from baseline in LDL cholesterol compared with placebo, and no significant changes were found in triglycerides and HDL cholesterol. Our study confirms these results. However, we also studied the effect of the addition of gestodene to unopposed estradiol, showing that the potentially adverse increase in triglycerides in the unopposed oral estradiol group was completely reversed by the addition of gestodene.

In the Heart and Estrogen/Progestin Replacement Study (HERS), special attention was focused on the role of Lp(a). In an analysis of subgroup interactions, Lp(a) was suggested to be one of the possible risk modifiers during the overall study. In the placebo group, the highest incidence of coronary heart disease (CHD) events was found in the quartile with the highest baseline levels of Lp(a). Among the patients treated, the absolute reduction induced in Lp(a) and also in the risk of CHD in the first year was greatest among those in the highest Lp(a) quartile. A greater reduction seemed to be associated with a decreased risk for myocardial infarction, although not with a decrease in mortality. A comparable correlation between baseline values of Lp(a) and the lowering effect of HT has been shown previously. In our study, the greatest reduction in Lp(a) levels was also found in women in the tertile with the highest baseline levels.

Our study has several limitations. As we wanted to give unopposed estradiol for a year, we included hysterectomized women only. As this may hamper the diagnosis of the postmenopausal state, E2 and FSH levels were measured twice during the screening period. Despite the fact that in the screening period estradiol levels were below 110 pmol/L, 13 women had levels above 150 pmol/L at baseline. However, reanalysis without these women showed highly similar results. Unfortunately, the median serum Lp(a) level at baseline was lower in the placebo and transdermal groups than in the oral treatment groups. Because this study was randomized, this difference is purely by chance; however, because of the relatively small study groups and the naturally large skewness of Lp(a), in advance, the chance of finding a significant difference was large. These differences were corrected
for in the ANCOVA. Therefore, in our opinion, our findings are independent of differences in baseline levels of estradiol and Lp(a) between the groups. Furthermore, diet and other life-style characteristics were not assessed by standardized methods, although all participants were instructed not to change their habits and concomitant medication during the study. If however, such changes would have occurred, the randomized design probably would have provided an equal division over all four groups.

Conclusion

This study shows that oral low-dose estradiol induces beneficial changes in the lipid profile and reduces Lp(a) levels in healthy postmenopausal women. These changes are fewer or absent after transdermal estradiol administration. Addition of gestodene does not attenuate, and may even augment, the estradiol-induced effect.

Acknowledgments

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Effects of transdermal and oral postmenopausal hormone therapy on vascular function: a randomized, placebo-controlled study in healthy postmenopausal women

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Abstract

Objective: To compare the effect of transdermal and oral estrogen therapy, the latter with or without the addition of gestodene, on plasma concentrations of markers of endothelial function and on ultrasonographic parameters of vascular function in healthy postmenopausal women.

Design: In a 15-month, randomized, double-blind, placebo-controlled study, 152 healthy hysterectomized postmenopausal women received daily doses of placebo (n=49), 50 μg of transdermal 17β-estradiol (E₂) (tE₂, n=33), 1 mg of oral E₂ (oE₂, n=37), or 1 mg of oral E₂ combined with 25 μg of gestodene (oE₂+G, n=33) for 13 cycles of 28 days, followed by four washout cycles with placebo in each group. At baseline and in cycles 4, 13 and 17, we measured plasma levels of endothelial markers and ultrasonographic markers of vascular function (pulsatility index [PI] and, at baseline and cycle 13, arterial stiffness).

Results: Compared with placebo, we found reductions in soluble vascular cell adhesion molecule (oE₂, P<0.01; oE₂+G, P<0.001), sE-selectin (oE₂+G, P<0.05), von Willebrand factor (tE₂, P<0.05), and divergent effects in PI and stiffness parameters in the carotid artery. We found no effect on PI in the retinal and femoral arteries, or on stiffness parameters in the femoral and brachial artery.

Conclusions: Oral hormone therapy reduced plasma levels of adhesion molecules, whereas transdermal estrogen therapy reduced von Willebrand factor. Effects on ultrasonographic parameters of vascular function in the carotid artery were inconclusive.
Introduction

Cardiovascular disease is a major health issue among women after menopause. Observational studies have shown a possible cardioprotective effect of postmenopausal hormone therapy (HT). Addition of a progestogen did not appear to attenuate the cardioprotective effects of postmenopausal estrogen therapy. The effects seen in these studies were supported by a large amount of evidence provided by randomized controlled studies showing favorable effects of various HT regimens on surrogate endpoints, especially the lipid profile. However, large randomized controlled trials with clinical endpoints, such as the Heart and Estrogen/Progestin Replacement Study (HERS) and the Women’s Health Initiative (WHI) trial could not confirm the cardioprotective effects found in observational studies and reported no reduction or even an early increase in coronary heart disease in women assigned to HT.

An often mentioned explanation for the results of the HERS and WHI trial is the use of the combination of conjugated equine estrogens (CEE) plus medroxyprogesterone acetate (MPA). It is known from other studies that different HT regimens may induce different effects, and in particular that progestogens can either oppose or enhance effects induced by estrogens. New research needs, therefore, to focus on the balance of favorable and adverse effects of other HT regimens and alternative administration routes. For example, when applied transdermally, estradiol bypasses the liver, so with fewer effects on metabolic processes. For gestodene, a so-called third generation progestogen, mainly known for its use in oral contraceptives, neutral or even potentially beneficial effects on cardiovascular markers have been shown when added to estradiol in HT.

Atherosclerosis has a central role in the development of cardiovascular disease, and is preceded by endothelial dysfunction. Various markers that can be measured in plasma or with ultrasound have been associated with atherosclerosis and an increased risk of cardiovascular disease. These markers can therefore be useful to evaluate the effects on vascular function of HT regimens different from CEE plus MPA.

Previous studies have shown potentially favorable effects of HT on plasma and ultrasonographic markers of vascular function, whereas others have reported no significant effects. Many studies however, were observational. Only a few studies have investigated the effects of unopposed transdermal or oral estrogen therapy in postmenopausal women on markers of early atherosclerosis in a direct comparison with an untreated or placebo control group. Many of these studies, however were short-term. Multiple combined regimens have been studied, but few data are available on the effect of gestodene, a progestogen recently used in HRT.

We designed this randomized, placebo-controlled, 1-year study to compare the long-term effects of transdermal and oral estradiol therapy on parameters of vascular function and to investigate the effect of addition of gestodene to oral estradiol. We measured plasma markers of endothelial function, and the pulsatility index (PI) and arterial stiffness in multiple vascular beds.
Methods

Participants

The study design has been described previously.\textsuperscript{7-10,31} We included 152 healthy postmenopausal women who had undergone a hysterectomy (not necessarily combined with an oophorectomy). After screening 363 women, 152 women were eligible and enrolled in this study, which was performed at the outpatient clinics of the Departments of Obstetrics and Gynecology of four hospitals in the Netherlands (Amsterdam, Nijmegen, Utrecht, and Enschede). The investigation conformed to the principles outlined in the Declaration of Helsinki. The Institutional Review Boards of all participating centers approved the protocol. Written informed consent was obtained from each participant before entry into the study.

All participants were between 45 and 65 years of age, were nonsmokers or smoked less than six cigarettes per day, had blood pressures below 160 mmHg systolic and 100 mmHg diastolic, and had a body mass index (BMI) of 30 kg/m\textsuperscript{2} or less. Postmenopausal status was defined as having serum follicle-stimulating hormone (FSH) concentrations above 40 IU/L and estradiol concentrations lower than 110 pmol/L on each of two different visits in the screening period. None of the women had received HT within 6 months before randomization and none took cardiovascular medication, lipid-lowering drugs or drugs known to influence estrogen or progestogen metabolism. Exclusion criteria included a history of cardiovascular, venous thromboembolic, metabolic, endocrinologic (except for thyroid disease in a stable phase), and (pre-) malignant disease (except for successfully resected basal skin cancer), as well as clinically relevant abnormalities in laboratory tests of hematologic, renal and hepatic function or glucose metabolism. Women with fasting plasma cholesterol levels above 8 mM were excluded as well.

A double-dummy approach was used to maintain blinding of the study medication for participants, clinical investigators, and laboratory personnel. Eligible women were randomly assigned to either a placebo tablet and placebo patch (placebo group; \(n=49\)), to 50 μg of transdermal 17β-estradiol (Climara\textsuperscript{a}) daily and a placebo tablet (tE\textsubscript{2} group, \(n=33\)), to 1 mg of oral micronized 17β-estradiol daily and a placebo patch (oE\textsubscript{2} group, \(n=37\)), or to 1 mg of oral micronized 17β-estradiol plus 25 μg of gestodene (one tablet) daily and a placebo patch (oE\textsubscript{2}+G group, \(n=33\)), given for the first 13 28-day cycles, followed by four cycles of placebo tablets and placebo patches for each group. Tablets were taken daily and patches were changed once every 7 days. Medication was manufactured, packaged and labeled by Schering AG (SBU Fertility Control & Hormone Therapy, Berlin, Germany). All tablets were identical in appearance and organoleptic characteristics, as were all patches. Computerized randomization was done in blocks, which had a size of 13 and consisted of four times placebo treatment and three times of each active treatment for the first 13 cycles plus placebo treatment for the last four cycles. We included more women in the placebo group than in the other groups, as we expected more dropouts in this group. The statistical department of Schering AG generated the allocation sequence. Enrollment of participants and assignment to the treatment was done by the investigator in each hospital. Medication boxes were numbered and allocation was done in sequence. The blinding was discontinued after all measurements were performed at the end of the study.

It was not possible to obtain sufficient blood samples in one woman in the oE\textsubscript{2}+G group during the study. Three women dropped out before the measurements in cycle 4. In one woman in the
oE₂+G group insufficient blood samples were obtained for von Willebrand factor (vWF) measurement in cycle 4. Between the measurement in cycle 4 and in cycle 13, seven women dropped out (Figure 1). In addition, it was not possible to obtain sufficient blood samples in cycles 13 and 17 in one woman in the oE₂+G group. Analyses of the first 13 cycles were based on 148 women by using the last-observation-carried-forward procedure.

After the measurements in cycle 13, when all women received placebo, another six women dropped out (Figure 1). In another woman in the oE₂ group, insufficient blood samples were obtained for endothelin-1 measurement in cycle 17. Therefore, the analysis of the washout period for women completing the trial was based on 133 women for endothelin-1 and on 134 women for the other endothelial markers.

Because of logistical circumstances it was not possible to perform all ultrasound measurements in all women. Whether a woman did participate in these measurements was strictly based on the center in which she participated in the study. PI measurements in the femoral artery were performed in three of the four centers (136 participants), and PI measurements in the retinal arteries and stiffness measurements in one center (66 participants). In some women it was not possible to perform measurements at each measurement point: data were incomplete for 8, 10, and 8 subjects in the carotid, femoral and retinal arteries, respectively. Vascular stiffness measurements using the Wall

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**Figure 1. Study outline**

n, number of participants; tE₂, transdermal 17β-estradiol; oE₂, oral 17β-estradiol; oE₂+G, oral 17β-estradiol plus gestodene
Track System were performed at baseline and in cycle 13 in one center only (VU University Medical Center); data were incomplete for 6, 9 and 12 subjects in the carotid, femoral and brachial artery, respectively.

**Endothelial function markers**

At baseline and in cycle 4 (cycle day 1 to 14), cycle 13 (cycle day 1 to 28) and cycle 17 (cycle day 1 to 28) of follow-up, venous blood samples were taken between 8.00 a.m. and 10.00 a.m., with participants in the supine position. The women had fasted for at least 10 hours and had refrained from smoking for at least 10 hours and from consuming alcohol for at least 24 hours. After 20 minutes of rest, blood was collected with a Vacutainer® system (Becton Dickinson, Meyren, Cedex, France) into pre-cooled tubes containing 0.105M sodium citrate for vWF, and K$_3$-EDTA for all other markers (Becton Dickinson, Plymouth, UK). Plasma was separated by centrifugation at 3,000 g at 4°C for 30 minutes within 1 hour of collection. Plasma was divided into aliquots, snap-frozen and stored at -80°C until analysis.

For measurement of plasma levels of soluble vascular cell adhesion molecule (sVCAM), inter-cellular cell adhesion molecule and sE-selectin we used commercially available enzyme-linked immunosorbent assays (Diaclone, Besançon, France). Endothelin-1 concentrations were determined using a standard commercially available high-sensitivity enzyme-linked immunosorbent assay (R&D systems, Oxford, UK) after extraction on Sep-Pak C18 cartridges (Waters). Plasma vWF antigen was measured by in-house sandwich enzyme immunoassay, using rabbit anti-vWF antigen immunoglobulin G as a catching antibody and a peroxidase-conjugated rabbit anti-vWF-antigen as detecting antibody (Dako, Copenhagen, Denmark). O-Phenylenediamine (Sigma Chemical Co., St. Louis, MO, USA) was used as substrate. Levels of vWF are expressed as a percentage of antigen levels in normal pooled plasma (%NPP), which is defined as 100%. All intra- and interassay coefficients of variation (CV) were less than or equal to 4.6% and 10.9%, respectively.

All samples of a given subject were assayed in duplicate within a single run.

**Ultrasound measurements**

Pulsatility index reflects resistance to flow distal to the measurement point. It is calculated from the Doppler waveforms from arteries as (S-D)/M, where S is the peak systolic velocity, D is end-diastolic velocity and M is the mean flow velocity during the cardiac cycle. As extremities have a higher resistance to flow, the shape of the waveform is different in peripheral arteries. Therefore, the resistance index (RI), calculated as (S-D)/S, was used in the femoral artery. Both variables increase with higher resistance to flow.

All color Doppler measurements were performed in a noiseless room with a constant temperature. Pulsatility and resistance index values were obtained from two measurements in both the left and right side artery; the calculated mean of these four values was used in the analyses. In the carotid artery, the PI was measured at five points: in the common carotid artery, proximal (just above the clavicle) and distal (1.5-2 cm proximal to the carotid bifurcation); in the internal carotid artery, proximal (1.5-2 cm distal to the carotid bifurcation) and distal (at the most distal visible point); and in the external carotid artery (1.5-2 cm distal to the carotid bifurcation). For the common and internal carotid artery, the calculated mean of the proximal and distal measurements was used for analyses. In the femoral artery the measurement points were 1-1.5 cm proximal to the bifurcation in
the common femoral artery and 2 cm distal to the bifurcation in the superficial and deep femoral artery. In the eye, we measured the ophthalmic artery (in the temporal area of the bulb), the central retinal artery (at its point of entry into the optic nerve) and the posterior ciliary artery (on the temporal aspect of the globe). For all measurement points the intra- and interobserver CV was less than 10.2%, except for the inter-observer CV in the ophthalmic artery, which was 12.4%.

The compliance and distensibility of the common carotid, the femoral and the brachial artery were determined by a noninvasive ultrasound technique. All women had fasted and refrained from smoking for more than 4 hours. One observer performed all measurements in a temperature-controlled room with a vessel wall movement detector (Wall Track System, Neurodata, Bilthoven, The Netherlands). This system consists of an ultrasound imager (Ultramark IV, ATL, Bothell, WA) connected to a data acquisition and processing unit. Using a 7.5-MHz linear array transducer in combination with the arterial wall movement detection system, we measured displacement of the anterior and posterior wall of the artery during the cardiac cycle with a precision in micrometers; the exact protocol has been described elsewhere.32

This ultrasound technique was used for the determination of common carotid, femoral and brachial artery diastolic diameter (D) and distension (Δd) during the cardiac cycle; when combined with pulse pressure (ΔP) measurements, compliance and distensibility coefficients were calculated. Compliance coefficient (CC) is defined as \((2*\pi*D*Δd+Δd^2)/(4*0.1333*ΔP)\), in \(\text{mm}^2/\text{kPa}\), and distensibility coefficient (DC) as \((2*Δd*D+Δd^2)/(0.133*ΔP*D^2)\), in \(10^{-3}/\text{Pa}\). Intraobserver intersession CV was as follows: CC 8.3% (carotid), 9.7% (femoral) and 15.6% (brachial); and DC 9.7%(carotid), 10.9% (femoral) and 16.1% (brachial).

Other measurements

Serum FSH was determined with a specific immunometrical (luminescence) assay (Amerlite, Amersham, Little Chalfont, UK). Serum estradiol was quantified by using a double-antibody radioimmunoassay (Sorin Biomedica, Saluggia, Italy) with a lower limit of detection of 18 pM. Serum total cholesterol was measured automatically (Boehringer Mannheim, Germany)

Statistics

Statistical analysis was performed using the Statistical Package for the Social Sciences PC 10.0 (SPSS Inc., Chicago, IL, USA). Values are given as mean and standard deviation or as median (25th–75th percentile), changes are given as mean (95% CI) or as geometric mean if the distribution was skewed. We compared measurements between groups using standard parametric tests; if the distribution was skewed, this was done after log transformation. Nonparametric tests were used when distribution remained skewed after log transformation. Analyses of covariance for repeated measurements with the baseline level of the parameter under consideration as covariate were used for comparisons among and between the groups. In series of repeated measurements (serum markers and PI) we used the last-observation-carried-forward procedure for the missing values at cycle 13 in eight women.

Although menopause was diagnosed after measuring serum estradiol (E2) and FSH levels twice during the screening period, 13 women had E2 levels above 150 mmol/L at baseline. Although the levels did not differ significantly among the treatment groups, the presence of these outliers prompted us to do additional analyses with correction for baseline E2 concentration; this
showed highly similar results. We show results of analyses of covariance with additional correction for baseline E$_2$.

Sample size calculation was based on the primary outcome variable of the original protocol (i.e. high-density lipoprotein cholesterol). To detect a mean difference of 0.2 mmol/L between the treatment groups and the placebo group with 80% power using a two-sided test at the 5 percent significance level, 36 evaluable women in each treatment arm were required.

Results

At baseline, the groups were comparable with respect to age, BMI, blood pressure, smoking habits, total cholesterol, serum FSH and E$_2$ levels (Table I) and alcohol consumption (data not shown). This was also the case for the subgroups of subjects that participated in the various ultrasonographic measurements (data not shown). Baseline values of the plasma markers (Table II) and of ultrasonographic parameters (Tables III and IV) did not reveal significant differences among the groups.

Markers of endothelial function and inflammation

After 13 cycles of treatment, we found reductions compared with placebo in sVCAM in the oE$_2$ group (P<0.05) and the oE$_2$+G group (P<0.001), in sE-selectin in the oE$_2$+G group (P<0.05) and in vWF in the tE$_2$ group (P<0.05) (Table II and Figure 2). There was a significant difference between all active treatment groups in the effect on sVCAM and between the oral treatment groups in the effect on sE-selectin.

Pulsatility and resistance indices

Some effects compared with placebo were observed in the PI of the carotid artery (Table III and Figure 3). In the common carotid artery, the PI was decreased in the tE$_2$ and oE$_2$ group (P<0.05); the oE$_2$+G group showed an increase in PI in both the internal (P<0.01) and external (P<0.05) carotid artery. We found no significant effects on resistance index in the femoral artery and PI in the retinal arteries.

Arterial stiffness

For a subgroup of women, we observed a percentage decrease in distension in the carotid artery in the tE$_2$ group (P<0.01), which was reflected in a decrease in the distensibility (P<0.01) and compliance (P<0.05) coefficient (Table IV and Figure 3). No effects on arterial stiffness parameters were observed in the femoral and brachial artery. In this subgroup, we also measured flow-mediated (endothelium-dependent) dilatation and nitroglycerin-induced (endothelium-independent) dilatation in the brachial artery; however, because of high variation coefficients of the measurements, these results were not interpretable.

Washout

Of the changes found in the plasma markers and the pulsatility index in the carotid artery in cycle 13, only the changes in the PI in the external carotid artery in the oE$_2$+G group persisted after 4 cycles of wash-out, all other effects had disappeared (data not shown).
Discussion

In this 1-year, randomized, placebo-controlled study, we found some potentially favorable but also neutral and some possibly unfavorable effects of three different hormone therapy regimens on multiple parameters of vascular function in healthy postmenopausal women. The observed changes were generally of low statistical significance, and the ultrasonographic measurements showed some divergence within the different treatment groups.

To gain insight in the effects of HT on the multiple processes involved in the development of cardiovascular disease, trials studying the effects on individual markers are needed. Such trials need to balance potentially favorable effects, such as improvement of the lipid profile,9 and adverse effects, such as changes in coagulation factors.7,8 Lowering levels of cardiovascular risk markers such as blood pressure and cholesterol can improve clinical outcome. The variables we investigated in this study are known to be associated with the development of coronary heart disease.16-18 However, the clinical relevance of a change in these variables during HT has yet to be established.

Adhesion molecules, such as VCAM, intercellular cell adhesion molecule, and E-selectin, play an important role in the attachment to and transendothelial migration of leukocytes through the vascular wall, thus facilitating the inflammatory process of atherosclerosis. Expressed by endothelial cells, they are shed into the blood in a soluble form.33 High concentrations correlate positively with high endothelial cell expression.33 The plasma levels of adhesion molecules in soluble form are considered a marker for atherosclerosis and cardiovascular disease.16,34 Endothelin-1 is a powerful vasoconstrictor and a marker of endothelial dysfunction; it up-regulates expression of adhesion molecules and opposes nitric oxide.35 Von Willebrand factor is a marker of generalized endothelial dysfunction and is increased in patients with peripheral and coronary artery disease.16

<table>
<thead>
<tr>
<th>Table I. Descriptive characteristics of the four groups at baseline</th>
</tr>
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<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Number of women</td>
</tr>
<tr>
<td>Age (years)</td>
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<tr>
<td>Body mass index (kg/m²)</td>
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<tr>
<td>Blood pressure:</td>
</tr>
<tr>
<td>systolic (mmHg)</td>
</tr>
<tr>
<td>diastolic (mmHg)</td>
</tr>
<tr>
<td>Smokers n (%)</td>
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<tr>
<td>Serum cholesterol (mmol/L)</td>
</tr>
<tr>
<td>Serum FSH (U/L)</td>
</tr>
<tr>
<td>Serum E₂ (pmol/L)</td>
</tr>
</tbody>
</table>

Values are given as mean ± SD, median (25th to 75th percentile), or as number (n) with percentage in parentheses. tE₂, 50 μg transdermal 17β-estradiol; oE₂, 1 mg oral 17β-estradiol; oE₂+G, oE₂ plus 25 μg gestodene; FSH, follicle-stimulating hormone; E₂, estradiol.

*One-way ANOVA or Kruskal-Wallis- or χ²-test for between-group differences.
Table II. Markers of endothelial function and inflammation

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Cycle 4</th>
<th>Cycle 13</th>
<th>Cycle 17</th>
<th>Pa</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>sVCAM (ng/mL)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>926 ± 157</td>
<td>941 ± 175</td>
<td>929 ± 170</td>
<td>&lt;0.001b</td>
<td>942 ± 187</td>
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<tr>
<td>tE2</td>
<td>891 ± 196</td>
<td>910 ± 214</td>
<td>900 ± 200</td>
<td>0.71</td>
<td>897 ± 180</td>
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<tr>
<td>oE2</td>
<td>879 ± 172</td>
<td>851 ± 157</td>
<td>858 ± 182</td>
<td>&lt;0.05c</td>
<td>884 ± 176</td>
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<tr>
<td>oE2+G</td>
<td>972 ± 197</td>
<td>884 ± 173</td>
<td>894 ± 176</td>
<td>&lt;0.001de</td>
<td>985 ± 197</td>
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<tr>
<td>Pf</td>
<td>0.14</td>
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</tr>
<tr>
<td><strong>sICAM (ng/mL)</strong></td>
<td></td>
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<tr>
<td>Placebo</td>
<td>505 ± 118</td>
<td>479 ± 113</td>
<td>487 ± 123</td>
<td>0.52b</td>
<td>519 ± 138</td>
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<tr>
<td>tE2</td>
<td>499 ± 104</td>
<td>462 ± 119</td>
<td>479 ± 98</td>
<td>0.58</td>
<td>485 ± 107</td>
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<tr>
<td>oE2</td>
<td>462 ± 94</td>
<td>431 ± 88</td>
<td>431 ± 96</td>
<td>0.16</td>
<td>454 ± 88</td>
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<tr>
<td>oE2+G</td>
<td>520 ± 184</td>
<td>474 ± 158</td>
<td>496 ± 144</td>
<td>0.29</td>
<td>524 ± 164</td>
</tr>
<tr>
<td>Pf</td>
<td>0.26</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>sE-selectin (ng/mL)</strong></td>
<td></td>
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<tr>
<td>Placebo</td>
<td>37 (27 to 51)</td>
<td>34 (25 to 45)</td>
<td>36 (26 to 50)</td>
<td>0.10b</td>
<td>39 (27 to 52)</td>
</tr>
<tr>
<td>tE2</td>
<td>42 (29 to 57)</td>
<td>36 (27 to 51)</td>
<td>39 (30 to 53)</td>
<td>0.84</td>
<td>43 (29 to 55)</td>
</tr>
<tr>
<td>oE2</td>
<td>35 (24 to 57)</td>
<td>32 (23 to 43)</td>
<td>32 (23 to 48)</td>
<td>0.42</td>
<td>36 (24 to 48)</td>
</tr>
<tr>
<td>oE2+G</td>
<td>31 (20 to 56)</td>
<td>27 (18 to 50)</td>
<td>30 (17 to 51)</td>
<td>&lt;0.05c</td>
<td>33 (20 to 49)</td>
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<td>Pf</td>
<td>0.50</td>
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<td><strong>Endothelin-1 (pg/mL)</strong></td>
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<td>Placebo</td>
<td>1.62 ± 0.35</td>
<td>1.54 ± 0.39</td>
<td>1.54 ± 0.37</td>
<td>0.35b</td>
<td>1.56 ± 0.40</td>
</tr>
<tr>
<td>tE2</td>
<td>1.57 ± 0.42</td>
<td>1.57 ± 0.37</td>
<td>1.57 ± 0.47</td>
<td>0.31</td>
<td>1.48 ± 0.47</td>
</tr>
<tr>
<td>oE2</td>
<td>1.47 ± 0.28</td>
<td>1.46 ± 0.36</td>
<td>1.38 ± 0.28</td>
<td>0.50</td>
<td>1.44 ± 0.32</td>
</tr>
<tr>
<td>oE2+G</td>
<td>1.43 ± 0.39</td>
<td>1.37 ± 0.29</td>
<td>1.42 ± 0.30</td>
<td>0.49</td>
<td>1.51 ± 0.35</td>
</tr>
<tr>
<td>Pf</td>
<td>0.08</td>
<td></td>
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<tr>
<td><strong>VWF (%NPP)</strong></td>
<td></td>
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</tr>
<tr>
<td>Placebo</td>
<td>106 ± 30</td>
<td>103 ± 27</td>
<td>105 ± 31</td>
<td>0.23b</td>
<td>102 ± 33</td>
</tr>
<tr>
<td>tE2</td>
<td>102 ± 33</td>
<td>100 ± 34</td>
<td>97 ± 31</td>
<td>&lt;0.05</td>
<td>102 ± 33</td>
</tr>
<tr>
<td>oE2</td>
<td>109 ± 30</td>
<td>108 ± 32</td>
<td>106 ± 32</td>
<td>0.29</td>
<td>110 ± 32</td>
</tr>
<tr>
<td>oE2+G</td>
<td>114 ± 41</td>
<td>112 ± 38</td>
<td>113 ± 37</td>
<td>0.68</td>
<td>112 ± 35</td>
</tr>
<tr>
<td>Pf</td>
<td>0.51</td>
<td></td>
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</table>

Concentrations are given as mean ± SD or median (25th to 75th percentile). tE2, 50 μg transdermal 17β-estradiol; oE2, 1 mg oral 17β-estradiol; oE2+G, oE2 plus 25 μg gestodene; s, soluble; VCAM, vascular cell adhesion molecule; ICAM, inter-cellular adhesion molecule; vWF, von Willebrand factor; NPP = normal pooled plasma.

a,bAnalysis of covariance for repeated measurements for between-group differences during 13 cycles with the baseline value of the variable under consideration and of baseline estradiol concentration as covariate in the treatment group versus placebo or overall, respectively. Pa<0.05 and Pa<0.001 versus tE2 group, and Pa<0.01 versus oE2 group.

fOne-way ANOVA or Kruskall-Wallis for between-group differences at baseline.
Figure 2. Percentage changes in cycle 13 in plasma markers

Values are given as (geometric) mean. For the comparison between groups: *P<0.05 and **P<0.01 versus placebo; †P<0.05 versus the tE2 group; and ‡P<0.001 versus the oE2 group. tE2, 50 μg transdermal 17β-estradiol; oE2, 1 mg oral 17β-estradiol; oE2+G, oE2 plus 25 μg gestodene; s, soluble; VCAM, vascular cell adhesion molecule; ICAM, inter-cellular adhesion molecule; vWF, von Willebrand factor.

Figure 3. Percentage changes in cycle 13 in markers of carotid artery function

Values are given as (geometric) mean. For the comparison between groups: *P<0.05 and **P<0.01 versus placebo; †P<0.05 versus the tE2 group; and §P<0.05 versus the oE2 group. tE2, 50 μg transdermal 17β-estradiol; oE2, 1 mg oral 17β-estradiol; oE2+G, oE2 plus 25 μg gestodene; PI, pulsatility index; common, common carotid artery; internal, internal carotid artery; external, external carotid artery; DC, distensibility coefficient; CC, compliance coefficient.
<table>
<thead>
<tr>
<th>Table III. Pulsatility and resistance indices</th>
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</thead>
<tbody>
<tr>
<td><strong>CAROTID ARTERIES</strong></td>
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<tr>
<td></td>
</tr>
<tr>
<td>Common</td>
</tr>
<tr>
<td>Placebo</td>
</tr>
<tr>
<td>tE&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>oE&lt;sub&gt;2&lt;/sub&gt;</td>
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<tr>
<td>oE&lt;sub&gt;2&lt;/sub&gt;+G</td>
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</tr>
<tr>
<td>oE&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>oE&lt;sub&gt;2&lt;/sub&gt;+G</td>
</tr>
<tr>
<td>Pd&lt;sup&gt;f&lt;/sup&gt;</td>
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<tr>
<td>Superficial</td>
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<tr>
<td>Placebo</td>
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<tr>
<td>tE&lt;sub&gt;2&lt;/sub&gt;</td>
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<tr>
<td>oE&lt;sub&gt;2&lt;/sub&gt;</td>
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<tr>
<td>oE&lt;sub&gt;2&lt;/sub&gt;+G</td>
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<td>Pd&lt;sup&gt;f&lt;/sup&gt;</td>
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<tr>
<td>Retinal</td>
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<tr>
<td>Placebo</td>
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<tr>
<td>tE&lt;sub&gt;2&lt;/sub&gt;</td>
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<tr>
<td>oE&lt;sub&gt;2&lt;/sub&gt;</td>
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<tr>
<td>oE&lt;sub&gt;2&lt;/sub&gt;+G</td>
</tr>
<tr>
<td>Pd&lt;sup&gt;f&lt;/sup&gt;</td>
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</table>

Values are given as mean ± SD. tE<sub>2</sub>, 50 μg transdermal 17β-estradiol; oE<sub>2</sub>, 1 mg oral 17β-estradiol; oE<sub>2</sub>+G, oE<sub>2</sub> plus 25 μg gestodene.

<sup>a,b</sup>Analysis of covariance for repeated measurements for between-group differences during 13 cycles with baseline value of the variable and of baseline estradiol concentration as covariate in the treatment group versus placebo or overall, respectively.

<sup>c</sup>P<0.05 versus oE<sub>2</sub>+G.

<sup>f</sup>One-way ANOVA for between-group differences at baseline.
Table IV. Arterial stiffness measurements

<table>
<thead>
<tr>
<th></th>
<th>CAROTID ARTERY</th>
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<td></td>
<td>Baseline</td>
<td>Cycle 13</td>
<td>P</td>
<td>Baseline</td>
<td>Cycle 13</td>
<td>P</td>
<td>Baseline</td>
<td>Cycle 13</td>
<td>P</td>
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<tr>
<td>Diameter (mm)</td>
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<tr>
<td>Placebo</td>
<td>6.5 ± 0.6</td>
<td>6.4 ± 0.6</td>
<td>0.90b</td>
<td>8.3 ± 0.9</td>
<td>8.0 ± 1.0</td>
<td>0.45b</td>
<td>3.4 ± 0.4</td>
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<td>tE2</td>
<td>6.5 ± 0.6</td>
<td>6.5 ± 0.6</td>
<td>0.60</td>
<td>7.8 ± 0.7</td>
<td>8.0 ± 0.6</td>
<td>0.58</td>
<td>3.4 ± 0.5</td>
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<td>oE2</td>
<td>6.5 ± 0.5</td>
<td>6.3 ± 0.5</td>
<td>0.83</td>
<td>7.9 ± 1.4</td>
<td>8.1 ± 1.0</td>
<td>0.95</td>
<td>3.2 ± 0.6</td>
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<tr>
<td>oE2+G</td>
<td>6.4 ± 0.5</td>
<td>6.4 ± 0.7</td>
<td>0.79</td>
<td>7.5 ± 1.1</td>
<td>7.6 ± 1.0</td>
<td>0.15</td>
<td>3.5 ± 0.6</td>
<td>3.2 ± 0.3</td>
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<tr>
<td>Pc</td>
<td>0.97</td>
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<td>Distension (μm)</td>
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<tr>
<td>Placebo</td>
<td>318 ± 80</td>
<td>337 ± 87</td>
<td>0.70b</td>
<td>286 ± 139</td>
<td>298 ± 96</td>
<td>0.84b</td>
<td>192 ± 95</td>
<td>197 ± 97</td>
<td>0.61b</td>
</tr>
<tr>
<td>tE2</td>
<td>353 ± 96</td>
<td>323 ± 87</td>
<td>0.52</td>
<td>274 ± 81</td>
<td>273 ± 89</td>
<td>0.92</td>
<td>189 ± 57</td>
<td>200 ± 66</td>
<td>0.81</td>
</tr>
<tr>
<td>oE2</td>
<td>334 ± 70</td>
<td>339 ± 76</td>
<td>0.63</td>
<td>302 ± 121</td>
<td>280 ± 110</td>
<td>0.66</td>
<td>205 ± 129</td>
<td>165 ± 86</td>
<td>0.42</td>
</tr>
<tr>
<td>oE2+G</td>
<td>358 ± 90</td>
<td>354 ± 83</td>
<td>0.24</td>
<td>265 ± 93</td>
<td>263 ± 89</td>
<td>0.64</td>
<td>175 ± 90</td>
<td>156 ± 63</td>
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<tr>
<td>Pc</td>
<td>0.51</td>
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<td>DC (1/kPa)</td>
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<td></td>
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<tr>
<td>Placebo</td>
<td>14.4 ± 3.5</td>
<td>16.1 ± 5.7</td>
<td>0.28b</td>
<td>10.7 ± 6.9</td>
<td>11.2 ± 4.3</td>
<td>0.85b</td>
<td>160 (9.0 to 25.7)</td>
<td>163 (9.4 to 24.1)</td>
<td>0.96b</td>
</tr>
<tr>
<td>tE2</td>
<td>14.7 ± 4.7</td>
<td>13.3 ± 3.6</td>
<td>0.48</td>
<td>9.4 ± 3.0</td>
<td>9.9 ± 4.5</td>
<td>0.64</td>
<td>13.4 (9.0 to 20.0)</td>
<td>14.2 (11.2 to 18.9)</td>
<td>0.78</td>
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<tr>
<td>oE2</td>
<td>15.4 ± 4.3</td>
<td>17.2 ± 5.1</td>
<td>0.43</td>
<td>11.7 ± 5.0</td>
<td>11.0 ± 4.8</td>
<td>0.65</td>
<td>16.9 (8.3 to 30.7)</td>
<td>15.8 (10.6 to 18.9)</td>
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<tr>
<td>oE2+G</td>
<td>17.2 ± 3.3</td>
<td>16.8 ± 4.4</td>
<td>0.21</td>
<td>11.1 ± 5.2</td>
<td>10.1 ± 3.4</td>
<td>0.99</td>
<td>13.6 (10.3 to 23.6)</td>
<td>11.9 (8.9 to 19.4)</td>
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<tr>
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<td>0.36</td>
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<tr>
<td>CC (mm²/kPa)</td>
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<tr>
<td>Placebo</td>
<td>0.47 ± 0.14</td>
<td>0.51 ± 0.20</td>
<td>0.34b</td>
<td>0.55 ± 0.29</td>
<td>0.54 ± 0.18</td>
<td>0.50c</td>
<td>0.14 (0.08 to 0.22)</td>
<td>0.14 (0.10 to 0.19)</td>
<td>0.61b</td>
</tr>
<tr>
<td>tE2</td>
<td>0.48 ± 0.14</td>
<td>0.43 ± 0.11</td>
<td>0.64</td>
<td>0.45 ± 0.14</td>
<td>0.47 ± 0.18</td>
<td>0.41</td>
<td>0.11 (0.09 to 0.16)</td>
<td>0.13 (0.11 to 0.15)</td>
<td>0.59</td>
</tr>
<tr>
<td>oE2</td>
<td>0.52 ± 0.16</td>
<td>0.52 ± 0.16</td>
<td>0.56</td>
<td>0.58 ± 0.28</td>
<td>0.56 ± 0.26</td>
<td>0.60</td>
<td>0.14 (0.09 to 0.22)</td>
<td>0.12 (0.09 to 0.18)</td>
<td>0.28</td>
</tr>
<tr>
<td>oE2+G</td>
<td>0.56 ± 0.13</td>
<td>0.55 ± 0.22</td>
<td>0.16</td>
<td>0.48 ± 0.23</td>
<td>0.46 ± 0.19</td>
<td>0.47</td>
<td>0.12 (0.09 to 0.17)</td>
<td>0.10 (0.08 to 0.18)</td>
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<tr>
<td>Pc</td>
<td>0.45</td>
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</table>

Values given as mean ± SD or median (25th to 75th percentile). DC, distensibility coefficient; CC, compliance coefficient; tE2, 50 μg transdermal 17β-estradiol; oE2, 1 mg oral 17β-estradiol; oE2+G, oE2 plus 25 μg gestodene.

*a,b*Analysis of covariance for repeated measurements for between-group differences during 13 cycles with baseline value of estradiol as covariate in the treatment group versus placebo or overall, respectively.

*c*One-way ANOVA or Kruskall-Wallis for between-group differences at baseline.
We found decreases in plasma levels of sVCAM in the oE2 and oE2+G groups, in sE-selectin in the oE2+G group and in vWF in the tE2 group. Considering the level of significance and the number of factors we studied, these findings may, however, be the result of chance. The observed decreases are in agreement with most previous studies that, compared with a control group, have reported a decrease in soluble adhesion molecule levels after short-term unopposed oral22,26 and transdermal22 estradiol therapy, after 1 year of oral estradiol plus gestodene therapy14 and by other combined estrogen-progestogen regimens.27 Other studies did not find any significant effect compared with a control group in endothelin-1 or vWF after short-term treatment with unopposed oral22,26 or transdermal22,23,25 estradiol therapy, nor after long-term combined HT.27 A decrease in soluble adhesion molecule levels would be consistent with the observed decreased in vitro expression of adhesion molecules on endothelial cells after 17β-estradiol exposure, with a greater effect after addition of a progestogen.36

Multiple ultrasound techniques exist nowadays to assess structure and function of the arterial wall in a noninvasive way. Subtle changes in the function of the vessel wall can be measured even before anatomical evidence of atherosclerosis is present.37 A decrease in PI reflects a decrease in resistance to flow in the artery distal to the measuring point and hence an increase in blood flow. The carotid artery has been studied in relation to cerebrovascular disease; the retinal and ciliary artery are end-arterial branches of the internal carotid artery and functionally and anatomically comparable to cerebral arteries of the same magnitude.38 Decreases in arterial distensibility and compliance reflect decreases in arterial elasticity and buffering capacity, respectively.

In the main branches of the carotid artery we found a decrease in PI in both unopposed estradiol groups compared to placebo, an increase in PI in the estradiol plus gestodene group, and a percentage decrease in compliance and distensibility in the transdermal group. These observed divergent effects in PI were of low statistical significance. Previous studies reported a decrease in PI in the carotid artery after transdermal19,39 or oral20 unopposed estradiol therapy compared with a control group, and no effect on arterial stiffness after long-term unopposed transdermal estradiol,24 oral estradiol plus gestodene,11 or combined CEE plus MPA.21

The clinical effect of various HT regimens is the result of the combination of multiple positive and negative effects on lipids, metabolic, haemostatic and inflammatory factors and on the vascular wall. Although surrogate endpoint studies have suggested a possible cardioprotective effect of various HT regimens in healthy postmenopausal women, the results of the HERS4 and WHI trial5 could not confirm this for CEE plus MPA in both healthy women and women with established coronary heart disease. Later studies using unopposed estrogen also could not find favorable effects of oral estradiol valerate40 and transdermal estradiol41 in women with established coronary heart disease, or of CEE42 in healthy late postmenopausal women. A cardioprotective effect in healthy women starting HT early after menopause has been suggested,42,43 but has yet to be established.

Our study had some limitations. Although it is not indicated to give a progestogen to women without a uterus, we included hysterectomized women only because about 50% of the women would be exposed to unopposed estradiol for 1 year. Because hysterectomy may hamper the diagnosis of the postmenopausal state, E2 and FSH levels were measured twice during the screening period. Although E2 levels in the screening period estradiol levels were below 110 pmol/L, 13 women had E2 levels above 150 pmol/L at baseline. Therefore, in our analysis of covariance, we corrected for both the baseline level of the variable under investigation and baseline estradiol levels. As baseline levels of
estradiol did not differ among the four treatment groups, results were highly similar with and without additional correction for baseline estradiol. Because the sample size for this study was calculated for a parameter (high-density lipoprotein cholesterol) which was not part of this sub-study, numbers may have been too small to achieve sufficient power to detect possible effects for some parameters.

**Conclusions**

In this randomized, placebo-controlled study, we found some potentially beneficial effects of three HT regimens on plasma markers of endothelial function and divergent effects on multiple ultrasonographic parameters of arterial function in the carotid artery. The cumulative effect of all changes remains inconclusive.

**Acknowledgments**

The authors wish to thank MS Post, MD, H Kessel, MD, and M Steyn, for data collection, ERA Peters-Muller, MSc, for performing statistical analyses, A Kok, for logistic laboratory management (VU University Medical Center, Amsterdam); and the following investigators: JMWM Merkus, MD, PhD, CPT Schijf, MD, CF van Heteren, MD, and JMJ Smeenk, MD (University Medical Center St. Radboud, Nijmegen), MVAM Kroeks, MD, PhD (Diakonessenhuis, Utrecht), HR Franke, MD, PhD (Medisch Spectrum Twente Hospital Group, Enschede); and all women who participated in the study.

**References**


Oral, more than transdermal, oestrogen therapy lowers asymmetric dimethylarginine in healthy postmenopausal women:
a randomized, placebo-controlled study

Marieke O Verhoeven
Majoie Hemelaar
Marius J van der Mooren
Peter Kenemans
Tom Teerlink

*Journal of Internal Medicine 2006;259:199-208*

The definitive version is available at:
http://www.blackwell-synergy.com
Abstract

**Objective:** To compare the effects of oral and transdermal hormone therapy (HT) on asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase, in postmenopausal women.

**Design:** In a multicentre, placebo-controlled, double-blind study, 152 hysterectomised healthy women were randomised to receive daily transdermal 17β-oestradiol (tE₂, n=33), or oral micronised 17β-oestradiol either unopposed (oE₂, n=37), or continuous combined with gestodene (oE₂+G, n=33), or placebo (n=49) for thirteen 28 day treatment cycles. Plasma concentrations of ADMA, arginine, and symmetric dimethylarginine (SDMA) were measured at baseline and in treatment cycles 4 and 13 with a high-performance liquid chromatography method.

**Results:** After 13 cycles all active treatment groups showed a significant reduction in ADMA compared with placebo: tE₂, -4.0% (95% CI -7.5 to -0.6%); oE₂, -7.7% (95% CI -10.9 to -4.4%); and oE₂+G, -7.5% (95% CI -10.8 to -4.3%). ANCOVA showed a significantly larger reduction in the oral groups compared with the transdermal group (tE₂ vs. oE₂ and tE₂ vs. oE₂+G, both P<0.01). Oral, but not transdermal treatment, significantly reduced arginine compared with placebo. All active treatments reduced SDMA; however, this was only statistically significant in the oE₂ group.

**Conclusion:** Reduction of ADMA was more pronounced after oral than after transdermal E₂ administration. Adding gestodene to oral 17β-oestradiol did not alter the reduction of ADMA. The clinical implications of these findings remain uncertain; however, the decrease of ADMA by 17β-oestradiol could be a key phenomenon in the modulation of nitric oxide synthesis by postmenopausal HT.
Introduction

Before the age of 50 years, the incidence of coronary heart disease (CHD) is more frequent in men than in women, whereas after menopause the risk for CHD increases exponentially in women.\textsuperscript{1,2} It has been suggested that endogenous female hormones protect premenopausal women from CHD. This has led to the hypothesis that hormone therapy (HT) given to postmenopausal women might protect them from CHD.

In observational studies lower rates of CHD were found in women using oestrogens, either unopposed or combined with progestogens, in comparison with women not using HT.\textsuperscript{3,4} However, two large randomised placebo-controlled trials did not confirm this.\textsuperscript{5-7} HT was given orally in these clinical trials.\textsuperscript{5-7} Trials with transdermal 17β-oestradiol, either alone or combined with a progestogen are rare. In a non-blinded, non-placebo-controlled, randomised study, a non-significant increase in CHD events was observed in the transdermal hormone-treated group in the first two years.\textsuperscript{8}

Women in these trials\textsuperscript{5-8} were not representative of the large group of early postmenopausal women treated with HT for climacteric symptom relief. The mean age of the women in these studies was above 60 years and 10-15 years after the mean age of menopause.\textsuperscript{5-8} Moreover, in two studies, participants had to have a medical history of CHD.\textsuperscript{5,8} Thus, the effect of HT on CHD in younger, healthy postmenopausal women remains unclear. The present study was designed to explore possible effects of transdermal and oral HT on several cardiovascular risk factors in comparison with placebo in younger healthy postmenopausal women.\textsuperscript{9-16}

An emerging cardiovascular risk marker of interest is asymmetric dimethylarginine (ADMA), which is an endogenous inhibitor of nitric oxide synthase (NOS).\textsuperscript{17} ADMA is a risk factor for acute coronary syndromes.\textsuperscript{18} Furthermore, high plasma concentrations of ADMA have been associated with an increased overall mortality and more cardiovascular events in specific patients groups.\textsuperscript{19,20}

Little is known about ADMA plasma concentration in women undergoing the menopausal transition. One study analysed endogenous oestradiol (E\textsubscript{2}) concentrations in 33 women with CHD (mean age 58 years) and in 17 women without CHD (mean age 54 years).\textsuperscript{21} A multiple linear stepwise regression analysis showed that ADMA concentrations were inversely related with endogenous E\textsubscript{2} concentrations.\textsuperscript{21} Until now, no longitudinal data of ADMA concentrations in women during the menopausal transition have been published.

Post et al.\textsuperscript{22} investigated the effect of oral 17β-oestradiol, either unopposed or sequentially combined with dydrogesterone or trimegestone on ADMA concentrations, whereas Teerlink et al.\textsuperscript{23} examined the effects of oral conjugated equine estrogens (CEE) and raloxifene. In both studies an HT-induced reduction in ADMA was observed.\textsuperscript{22,23} So far, no studies on transdermal HT and ADMA have been published.

Therefore, the aim of this study was to compare, in a randomized placebo-controlled study, the effect of transdermal and oral 17β-oestradiol on plasma ADMA concentrations. Because it is known that the addition of progestogens can modulate the effect of oestrogens on metabolic and cardiovascular markers,\textsuperscript{22,24} the effect of the addition of the progestogen gestodene to oral 17β-oestradiol was investigated as well. Additionally, this study investigated the effects on plasma concentrations of arginine, a precursor of nitric oxide (NO), and symmetric dimethylarginine (SDMA), a stereoisomer of ADMA that does not inhibit NO synthase (NOS). The ratio between arginine and ADMA is considered important for NOS activity. Supplementation of L-arginine can reduce the inhibiting effect
of ADMA on NOS and partly restore NO production. This is why the arginine/ADMA ratio was calculated as well in this study. Previously, we reported on a variety of CHD risk markers measured in this trial. We were thus able to study correlations between ADMA and total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, homocysteine, C-reactive protein (CRP), soluble vascular cell adhesion molecule-1 (sVCAM-1), soluble intercellular adhesion molecule-1 (sICAM-1) and soluble E-selectin (sE-selectin) as well.

Materials and methods

Subjects

The design of this study has been published previously. Briefly, after screening, 152 eligible healthy postmenopausal women who had undergone a hysterectomy were enrolled in this multicentre study. The investigation conformed to the principles outlined in the Declaration of Helsinki. All Institutional Review Boards approved the protocol. Written informed consent was obtained from each participant before entering the study.

Participants were between 45 and 65 years old, smoked less than 6 cigarettes per day, had blood pressures below 160/100 mmHg and had a body mass index (BMI) of 30 kg/m² or less. Postmenopausal status was defined as a serum follicle-stimulating hormone (FSH) concentration above 40 IU/L and an endogenous E₂ concentration lower than 110 pmol/L on each of two different visits in the screening period. Having climacteric symptoms was not an inclusion or exclusion criterion. Only women requiring hormone treatment because of severe climacteric symptoms were excluded from this study. None of the women had received HT within 6 months before randomisation and none took cardiovascular medication. Exclusion criteria included a history of cardiovascular, thrombo-embolic, metabolic, endocrinological, and malignant disease, as well as clinically relevant abnormalities in laboratory tests.

To maintain blinding of the study medication, a double-dummy approach was used. Eligible women were randomly assigned to either a placebo tablet and placebo patch (placebo group, n=49), or to transdermal 17β-oestradiol 50 μg daily (Climara®) and a placebo tablet (tE₂ group, n=33), or to oral micronised 17β-oestradiol 1 mg daily and a placebo patch (oE₂ group, n=37), or to oral micronised 17β-oestradiol 1 mg and gestodene 25 μg daily (one tablet) and a placebo patch (oE₂+G group, n=33) given for 13, 28-day cycles. Medication was manufactured by Schering AG (SBU Fertility Control & Hormone Therapy, Berlin, Germany). We included more women in the placebo group than in the other groups, as we expected more dropouts in this group.

At the visits during the treatment period the women returned the surplus patches and tablets, which were counted for compliance evaluation. Women were considered non-compliant if they returned more than 10% of the number of patches and tablets dispensed. According to this criterion, five women (less than 4%) were non-compliant (placebo, n=2; tE₂, n=2; oE₂+G, n=1). Re-analysis excluding these women did not alter the results (data not shown).

Blood sampling

At baseline, in cycle 4 (cycle day 1 to 14) and cycle 13 (cycle day 1 to 28) venous blood samples were taken between 8:00 and 10:00 a.m., with participants in a supine position. The subjects had
fasted and refrained from smoking for at least 10 hours and from consuming alcohol for more than 24 hours. After 20 minutes of rest, blood was collected with a Vacutainer® system (Becton Dickinson, Meyren, Cedex, France) into cooled tubes containing tri-potassium ethylenediaminetetra-acetic acid (K3EDTA) (Becton Dickinson, Meylan, Cedex, France). Blood samples were immediately placed on ice and plasma was separated by centrifuging at 3,000 g and 4ºC for 30 minutes within 1 hour of collection. Plasma was divided into aliquots, snap-frozen and stored at -80°C until analysis.

**Laboratory methods**

ADMA, arginine, and SDMA were measured by high-performance liquid chromatography with fluorescence detection. All samples from individual patients were analysed in the same analytical series. The intra-assay and inter-assay coefficients of variation for all analyses were less than 1.2% and 3%, respectively. For each participant the plasma arginine/ADMA ratio was calculated.

The analyses of FSH, endogenous E2, lipids, homocysteine, CRP and adhesion molecules were described previously.9,10,14,16

**Statistics**

Statistical analysis was performed using the Statistical Package for the Social Sciences PC + 10.0.5 (SPSS Inc., Chicago, IL, USA). Baseline characteristics are given as mean ± standard deviation when normally distributed or as median (range) when the distribution was skewed. ADMA, arginine and SDMA concentrations and the arginine/ADMA ratio are given as mean ± standard deviation. Standard parametric tests were performed. Analyses of covariance (ANCOVA) for repeated measurements, with the baseline value of the variable under consideration, endogenous E2 concentration and smoking as constant covariates, were used for comparisons among and between the groups. The mean values of the individual percentage changes from baseline are given with 95% confidence interval (CI). A two-tailed P<0.05 was accepted as the level of significance.

No blood samples were available of seven women (placebo, n=1; tE2, n=1; oE2, n=3 and oE2+G, n=2). Furthermore, three women of whom only baseline samples were available (placebo, n=2 and tE2, n=1) and one woman, who had no data at baseline (placebo, n=1), were excluded from the analyses. For the missing results in another nine women in cycle 13, the last-observation-carried-forward procedure was applied using the results obtained at cycle 4 (placebo, n=3; tE2, n=2; oE2, n=2 and oE2+G, n=2). Therefore, the analyses of ADMA and arginine were based on 141 women (placebo, n=45; tE2, n=31; oE2, n=34; and oE2+G, n=31). In view of the fact of a lack of material, the results for SDMA were not obtainable in several samples and as a result the analyses of SDMA were based on 137 women (placebo, n=44; tE2, n=29; oE2, n=33; and oE2+G, n=31 women).

Associations of age, BMI, blood pressure, FSH, and endogenous E2 with baseline concentrations of ADMA were assessed by calculating Pearson’s correlation coefficient. Furthermore, correlations were calculated between baseline values of ADMA and baseline values of arginine, SDMA, and several other relevant factors, measured previously in this study (total cholesterol, LDL and HDL cholesterol, triglycerides, homocysteine, CRP, sVCAM-1, sICAM-1 and sE-selectin). Correlations between the absolute changes in ADMA and the absolute changes in these factors after 13 cycles of treatment were calculated only in the combined active treatment groups. For these post-hoc analyses the Bonferroni P-correction for multiple comparisons was applied. The accepted significance level here was: P = 0.05/26 = 0.002.
Results

The trial profile, with the number of women in each treatment group at baseline and at cycles 4 and 13, is shown in Figure 1. Demographic characteristics of the sub-population analysed (n=141) did not differ significantly from the original population of 152 women. At baseline, no significant differences were found between the groups in either demographic characteristics or in any of the variables investigated (Table I). Although all women had endogenous E2 concentrations below 110 pmol/L twice during screening, 13 women had endogenous E2 concentrations above 150 pmol/L at baseline. These women were distributed as follows: four women in the placebo group (175, 246, 396 and 402 pmol/L); four women in the tE2 group (170, 207, 264 and 912 pmol/L); one woman in the oE2 group (192 pmol/L); and four women in the oE2+G group (153, 169, 173 and 333 pmol/L). Re-analysis without these women did not change the results (data not shown). Therefore, these women were included in the analyses. To correct for potential confounding by differences in baseline endogenous E2 concentrations and smoking habits (placebo, n=7; tE2, n=1; oE2, n=3; oE2+G, n=4; P=0.36, Fisher’s exact test), we added these two factors as well as the baseline value of the variables investigated into the ANCOVA as constant covariate.

Neither (serious) adverse events nor reasons for drop-out were associated with venous thromboembolic disease. Three women reported an episode of chest pain (tE2 group, n=1; oE2 group, n=1; oE2+G group, n=1) of whom one had developed hypertension as well (tE2 group). In one woman coronary ischemia was excluded by electrocardiography (oE2+G group), whereas in the other two women this could not be excluded with certainty.

Table II provides plasma concentrations of ADMA, arginine, SDMA and the arginine/ADMA ratio at the different time points. ANCOVA revealed significant reductions in ADMA concentrations in all active treatment groups (Table II). The mean individual percentage change versus baseline compared with placebo in the tE2 group reached statistical significance only at cycle 13, whilst the reduction in both oral groups was already statistically significant at cycle 4 (Figure 2). ANCOVA showed a significantly larger reduction in both oral groups compared with the transdermal group (tE2 versus oE2, P=0.004 and tE2 versus oE2+G, P=0.002).

Compared with placebo, 13 cycles of treatment with oE2 and oE2+G, but not with tE2 were associated with statistically significant decreases in arginine (Table II and Figure 2). Over the 13-cycle period there were no significant changes in SDMA levels. Only the percentage change in the oE2 group reached statistical significance (Table II and Figure 2) whilst ANCOVA showed no significant difference among and between groups in the arginine/ADMA ratio.

At baseline, ADMA concentrations were only significantly associated with arginine concentrations (r=0.30; P<0.001). The absolute changes in ADMA at cycle 13 correlated with the changes in arginine and SDMA (both r=0.36; P=0.001). The baseline concentrations of ADMA did not correlate with the baseline characteristics or the baseline values of any of the CHD markers that were measured previously in this study, i.e. lipids, homocysteine, CRP and adhesion molecules. In addition, the absolute change in ADMA concentration at cycle 13 was not correlated with any of the absolute changes of these CHD markers.
Figure 1. Clinical trial profile

The numbers of women in the upper section outlined with the dotted line are the women allocated to the four groups of the original population at baseline. The numbers of women in the lower outlined section are the women belonging to the four groups of the sub-population in which ADMA, arginine and SDMA were measured at baseline, cycle 4 and cycle 13.

tE₂, transdermal 17β-oestradiol 50 μg; oE₂, oral 17β-oestradiol 1 mg; oE₂+G, oE₂ plus gestodene 25 μg

Table I. Descriptive characteristics of the four groups at baseline

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>tE₂</th>
<th>oE₂</th>
<th>oE₂+G</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>45</td>
<td>31</td>
<td>34</td>
<td>31</td>
</tr>
<tr>
<td>Age (years)</td>
<td>55.2 ± 4.7</td>
<td>55.2 ± 4.8</td>
<td>54.2 ± 4.2</td>
<td>53.3 ± 4.2</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>25.6 ± 3.0</td>
<td>26.0 ± 2.4</td>
<td>24.9 ± 3.2</td>
<td>26.0 ± 2.9</td>
</tr>
</tbody>
</table>
| Blood pressure:
  systolic (mmHg)     | 128 ± 17 | 134 ± 14 | 127 ± 13 | 127 ± 17 |
  diastolic (mmHg)    | 79 ± 8   | 83 ± 9  | 81 ± 8  | 80 ± 9  |
| Smokers n (%)        | 7 (15)   | 1 (3)  | 3 (9)  | 4 (13) |
| Serum cholesterol (mmol/L) | 5.8 ± 0.8 | 6.1 ± 0.9 | 6.2 ± 1.0 | 6.0 ± 1.0 |
| Serum FSH (U/L)      | 63 ± 20  | 62 ± 22 | 65 ± 24 | 67 ± 33 |
| Serum endogenous E₂ (pmol/L) | 28 (18-402) | 28 (18-912) | 29 (18-192) | 29 (18-333) |

Values are given as number (n) with percentage in parentheses, as mean ± standard deviation, or as median (range). There were no statistically significant differences between the four groups in the baseline characteristics.

tE₂, transdermal 17β-oestradiol 50 μg; oE₂, oral 17β-oestradiol 1 mg; oE₂+G, oE₂ plus gestodene 25 μg; FSH, follicle-stimulating hormone; E₂, 17β-oestradiol.
### Table II. Concentrations of ADMA, arginine, SDMA, and the arginine/ADMA ratio during 13 treatment cycles

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Cycle 4</th>
<th>Cycle 13</th>
<th>ANCOVA&lt;sup&gt;d&lt;/sup&gt;</th>
<th>ANCOVA&lt;sup&gt;e&lt;/sup&gt;</th>
<th>% Δ 0 - 4&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% Δ 0 - 13&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ADMA (μmol/L)</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>0.45 ± 0.04</td>
<td>0.46 ± 0.05</td>
<td>0.46 ± 0.05</td>
<td>&lt; 0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tE&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.45 ± 0.04</td>
<td>0.45 ± 0.04</td>
<td>0.43 ± 0.03</td>
<td>0.045</td>
<td>-0.8 (-4.3 to 2.6)</td>
<td>-4.0 (-7.5 to -0.6)</td>
<td></td>
</tr>
<tr>
<td>oE&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.44 ± 0.04</td>
<td>0.42 ± 0.04</td>
<td>0.41 ± 0.04</td>
<td>&lt; 0.001</td>
<td>-6.3 (-9.9 to -2.7)</td>
<td>-7.7 (-10.9 to -4.4)</td>
<td></td>
</tr>
<tr>
<td>oE&lt;sub&gt;2&lt;/sub&gt;+G</td>
<td>0.47 ± 0.05</td>
<td>0.43 ± 0.05</td>
<td>0.43 ± 0.05</td>
<td>&lt; 0.001</td>
<td>-8.1 (-11.6 to -4.5)</td>
<td>-7.5 (-10.8 to -4.3)</td>
<td></td>
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<tr>
<td>P-value&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.18</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><strong>Arginine (μmol/L)</strong></td>
<td></td>
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<tr>
<td>Placebo</td>
<td>99.9 ± 17.4</td>
<td>98.6 ± 17.7</td>
<td>103.6 ± 18.8</td>
<td>&lt; 0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tE&lt;sub&gt;2&lt;/sub&gt;</td>
<td>96.2 ± 12.1</td>
<td>97.4 ± 12.2</td>
<td>95.6 ± 11.4</td>
<td>0.67</td>
<td>2.6 (-2.0 to 7.2)</td>
<td>-2.4 (-7.9 to 3.0)</td>
<td></td>
</tr>
<tr>
<td>oE&lt;sub&gt;2&lt;/sub&gt;</td>
<td>98.9 ± 13.7</td>
<td>96.0 ± 13.0</td>
<td>94.4 ± 14.0</td>
<td>0.03</td>
<td>-1.2 (-6.3 to 3.9)</td>
<td>-7.9 (-12.5 to -3.3)</td>
<td></td>
</tr>
<tr>
<td>oE&lt;sub&gt;2&lt;/sub&gt;+G</td>
<td>97.0 ± 12.4</td>
<td>88.2 ± 11.5</td>
<td>89.6 ± 13.3</td>
<td>&lt; 0.001</td>
<td>-7.9 (-12.1 to -3.7)</td>
<td>-10.6 (-15.3 to -5.9)</td>
<td></td>
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<tr>
<td>P-value&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.68</td>
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<tr>
<td><strong>SDMA (μmol/L)</strong></td>
<td></td>
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<tr>
<td>Placebo</td>
<td>0.47 ± 0.07</td>
<td>0.47 ± 0.08</td>
<td>0.48 ± 0.07</td>
<td>0.06</td>
<td></td>
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<tr>
<td>tE&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.45 ± 0.05</td>
<td>0.44 ± 0.05</td>
<td>0.44 ± 0.06</td>
<td>0.09</td>
<td>-1.3 (-6.4 to 3.9)</td>
<td>-5.2 (-11.1 to 0.7)</td>
<td></td>
</tr>
<tr>
<td>oE&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.46 ± 0.07</td>
<td>0.44 ± 0.08</td>
<td>0.44 ± 0.07</td>
<td>0.01</td>
<td>-4.2 (-9.2 to 0.9)</td>
<td>-6.4 (-12.0 to -0.8)</td>
<td></td>
</tr>
<tr>
<td>oE&lt;sub&gt;2&lt;/sub&gt;+G</td>
<td>0.47 ± 0.05</td>
<td>0.45 ± 0.07</td>
<td>0.46 ± 0.07</td>
<td>0.10</td>
<td>-2.9 (-7.7 to 1.8)</td>
<td>-5.1 (-10.2 to 0.0)</td>
<td></td>
</tr>
<tr>
<td>P-value&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.47</td>
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<tr>
<td><strong>Arginine/ADMA ratio</strong></td>
<td></td>
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<tr>
<td>Placebo</td>
<td>220 ± 34</td>
<td>216 ± 31</td>
<td>224 ± 35</td>
<td>0.06</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>tE&lt;sub&gt;2&lt;/sub&gt;</td>
<td>216 ± 26</td>
<td>219 ± 23</td>
<td>223 ± 27</td>
<td>0.41</td>
<td>3.3 (-2.0 to 8.6)</td>
<td>1.7 (-3.7 to 7.1)</td>
<td></td>
</tr>
<tr>
<td>oE&lt;sub&gt;2&lt;/sub&gt;</td>
<td>224 ± 35</td>
<td>232 ± 38</td>
<td>229 ± 33</td>
<td>0.09</td>
<td>5.2 (-0.4 to 10.8)</td>
<td>-0.1 (-5.1 to 4.2)</td>
<td></td>
</tr>
<tr>
<td>oE&lt;sub&gt;2&lt;/sub&gt;+G</td>
<td>211 ± 34</td>
<td>208 ± 35</td>
<td>208 ± 33</td>
<td>0.31</td>
<td>0.1 (-4.9 to 5.2)</td>
<td>-3.3 (-8.2 to 1.7)</td>
<td></td>
</tr>
<tr>
<td>P-value&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.37</td>
<td></td>
<td></td>
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</tbody>
</table>

Concentrations and ratios are given as mean ± standard deviation. % Δ, percentage changes and are given as mean (95% confidence interval) of the individual percentage changes from baseline compared with placebo at 4<sup>cycle</sup> 4 and at 4<sup>cycle</sup> 13. ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine; tE<sub>2</sub>, transdermal 17β-oestradiol 50 μg; oE<sub>2</sub>, oral 17β-oestradiol 1 mg; oE<sub>2</sub>+G, oE<sub>2</sub> plus gestodene 25 μg.

<sup>c</sup>One-way ANOVA, cross-sectional comparison for among-group differences.

<sup>d</sup>Analysis of covariance for repeated measurements (ANCOVA) for among-group differences with the baseline value of the variable under consideration, and baseline oestradiol concentrations and smoking as constant covariates, over the 13-cycle study period.

<sup>e</sup>ANCOVA over the 13-cycle period: placebo versus treatment. Other significant between-group differences were found for ADMA between the tE<sub>2</sub> and oE<sub>2</sub> group (P=0.004), and between the tE<sub>2</sub> and oE<sub>2</sub>+G group (P=0.002), for arginine between the tE<sub>2</sub> and oE<sub>2</sub>+G group (P<0.001) and between the oE<sub>2</sub> and oE<sub>2</sub>+G group (P=0.02).
Discussion

This randomised, placebo-controlled trial showed that transdermal as well as oral 17β-oestradiol therapy effectively reduced plasma ADMA concentrations after 13 cycles of treatment. Oral administration of 17β-oestradiol induced a larger decrease in ADMA concentrations than transdermal administration. Gestodene added continuously to oral 17β-oestradiol did not modify the oE2-induced change in ADMA concentrations. Compared with placebo, both oral treatment regimens reduced arginine concentrations significantly, whereas transdermal 17β-oestradiol did not. Whilst the reduction of SDMA in the three treatment groups was comparable, only the reduction in the oE2 group reached statistical significance at cycle 13.

The average reduction versus placebo in ADMA induced by oral 17β-oestradiol 1 mg, either given unopposed or continuously combined with gestodene, observed in this study (≈ 7%) is similar to the reduction found earlier after treatment with oral 17β-oestradiol 2 mg (≈ 5%) and oral CEE 0.625 mg treatment (≈ 7%) published previously by our group.22,23 In these two previous studies and the present study, ADMA concentrations were significantly reduced by oral estrogens to a similar
extent, indicating that the moderate ADMA lowering effect of estrogens is not a spurious finding but a real phenomenon. Although the absolute effect may seem small, it is noteworthy that the biological variation of plasma ADMA concentrations is also very low, with an interindividual coefficient of variation of approximately 10%. In the study by Valkonen et al.\textsuperscript{18} it was shown that even slightly increased ADMA concentrations (above 0.62 μmol/L) were associated with a strongly elevated risk for acute coronary events. Therefore, in our opinion the moderate reductions in ADMA levels as observed in the present and previous studies may be of clinical relevance.

Adding gestodene to oral 17β-oestradiol did not modify the effect on ADMA concentrations. In a previous study, we observed that trimetostone strongly enhanced the ADMA-lowering effect of oral 17β-oestradiol (21% versus 5%), whilst the addition of the progestogen dydrogesterone had no additional effect.\textsuperscript{22} All three progestogens have a strong affinity for the progesterone receptor. However, they probably differ in their trans-activation effects.\textsuperscript{27-29} This might explain part of the observed differences in the effects of the three progestogens on the 17β-oestradiol-induced changes in ADMA.

Both ADMA and SDMA are formed by methylation of arginine residues in proteins by a family of protein arginine methyltransferases (PRMTs). Free ADMA and SDMA, as measured in this study, are released upon proteolysis of these methylated proteins. SDMA is mainly cleared by renal excretion, whereas only a small part of ADMA is cleared from the circulation by this pathway. An increase in glomerular filtration rate as a cause for the ADMA-lowering effect of HT can thus be excluded, because this would affect SDMA concentrations to a larger extent than ADMA concentrations. Approximately 80% of ADMA is metabolized by the widely expressed enzyme dimethylarginine dimethylaminohydrolase (DDAH).\textsuperscript{30} DDAH is very sensitive to oxidative stress because the active site of the enzyme contains a critical sulfhydryl group that is required for its catalytic activity.\textsuperscript{31} Consequently, pathologic stimuli that induce oxidative stress, such as hyperhomocysteinemia\textsuperscript{32} and oxidized LDL,\textsuperscript{33} may reduce DDAH activity and lead to an accumulation of ADMA. Conversely, compounds with anti-oxidant properties, like oestrogens, may protect DDAH from inactivation by oxidants, leading to a reduction of ADMA concentrations.

Several in vitro and in vivo studies on the effect of oestrogens on ADMA metabolism have been conducted. Treatment of rats with 17β-oestradiol was shown to attenuate the increase in plasma ADMA concentrations after injection of LDL.\textsuperscript{34} Exposure of human and murine endothelial cells to 17β-oestradiol resulted in an increase in the activity of DDAH and was accompanied by a reduced release of ADMA.\textsuperscript{35} All in all, it seems plausible that HT causes an increase of DDAH activity, either directly or indirectly, i.e. by lowering LDL cholesterol and/or homocysteine concentrations. Although in the present study LDL cholesterol was significantly reduced after 13 cycles of treatment,\textsuperscript{14} absolute reductions of ADMA and LDL cholesterol were not correlated.

No correlations were found for baseline concentrations of ADMA and homocysteine or the absolute changes after 13 cycles of both variables. It is possible that in comparison with homocysteine other parameters are more dominant in affecting ADMA levels. Results obtained in studies looking at the course of ADMA concentrations after oral methionine loading are conflicting.\textsuperscript{36-39} Although an increased activity of DDAH is consistent with the results of the present study and earlier published data, it affords no explanation for the slight decrease of SDMA concentrations. Although only significant in the oE\textsubscript{2} group, the decrease in the other active treatment groups was of a similar magnitude. A similar non-significant decrease of SDMA concentrations was previously
observed by Post at al.\textsuperscript{22} and by Holden et al.,\textsuperscript{35} suggesting that the reduction of SDMA by HT is a real effect. Oxidative stress may lead to accelerated proteasomal degradation of proteins. It is thus conceivable that reduction of oxidative stress by HT, by reducing proteolysis of methylated proteins, leads to a diminished generation of both ADMA and SDMA. The combination of a decreased proteolysis and enhanced DDAH activity would explain the decrease of both ADMA and SDMA, the reduction of ADMA being more pronounced.

Recently, we have shown in both rats and humans that the liver plays an important role in the elimination of ADMA, probably through the degradation of ADMA by DDAH.\textsuperscript{40,41} This may provide an explanation for the observed difference between the transdermal and oral routes of HT administration. Unlike oral oestrogens, transdermally administered estrogens directly enter the systemic circulation without a first pass through the liver. The larger effect of oral therapy compared to transdermal thus confirms the crucial role of the liver in the elimination of ADMA and is in agreement with stimulation of DDAH activity by HT.

A major strength of this study is the randomised placebo-controlled double-blind design. In addition, the method used for measurement of methylated arginines has a very low coefficient of variation, allowing the reliable determination of relatively small treatment effects. As we wanted to give unopposed E\textsubscript{2} for a year, we included hysterectomised women only. As this may hamper the diagnosis of the postmenopausal state, E\textsubscript{2} and FSH concentrations were measured twice during the screening period. In spite of this, at baseline 13 women had E\textsubscript{2} concentrations above 150 pmol/L. It is possible that these women were perimenopausal. However, re-analysis without these women showed similar results. Therefore, we did not exclude these women, but corrected for baseline E\textsubscript{2} concentrations in the analysis of covariance. If a potential influence would exist, than it would have reduced the observed treatment effect. Another possible limitation could be that the number of women in this study was too small to permit proper assessment of associations between ADMA and other biochemical variables.

The ADMA was reduced by oral, and to a lesser extent by transdermal E\textsubscript{2} therapy. Although high levels of ADMA are associated with an increase in CHD events, the clinical implications of these ADMA reductions by 17β-oestradiol are at present unclear and need further investigation.

Acknowledgments

The authors wish to thank Mrs MS Post, MD and Mrs H Kessel, MD, for excellent logistical assistance and recruitment of participants (VU University Medical Center, Amsterdam), and the following investigators who participated in this study: JMWM Merkus, MD, PhD, CPT Schijf, MD, Mrs CF van Heteren, MD, Mrs JM Smeenk, MD (University Medical Centre Sint Radboud, Nijmegen); MVAM Kroeks, MD, PhD (Diakonessenhuis, Utrecht); HR Franke, MD, PhD (Medisch Spectrum Twente Hospital Group, Enschede); Mrs S de Jong for performing laboratory analyses, and all women who participated in the study.

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