Part II
Intranasal hormone therapy
Intranasal continuous combined 17β-estradiol/norethisterone therapy improves the lipid profile in healthy postmenopausal women

Majoie Hemelaar
Peter Kenemans
Lut de Bie
Peter HM van de Weijer
Marius J van der Mooren

Fertility and Sterility 2006;85:979-988

The definitive version is available at:
http://www.fertstert.org
http://www.fertstert.org/article/PIS0015028205041075
Abstract

**Objective:** To compare the effects of continuous combined 17β-estradiol (E2) plus norethisterone (acetate) [NET(A)] therapy by either intranasal or oral administration on the lipid profile in postmenopausal women.

**Design:** Randomized, double-blind, multicenter trial.

**Setting:** Gynecologic outpatient department.

**Patient(s):** Two-hundred and thirty-three healthy postmenopausal women.

**Intervention(s):** Women received continuous combined hormone therapy, either intranasal E2/NET 175 μg/275 μg as a spray (n=117), or oral E2/NETA 1 mg/0.5 mg as a capsule (n=116) for 1 year.

**Main outcome measure(s):** Fasting plasma concentrations of lipids and (apo)lipoproteins, and atherogenic indices at baseline and after 12, 24 and 52 weeks of treatment.

**Result(s):** We found a significant (P<0.001) decrease from baseline in both treatment groups in total, low-density lipoprotein (LDL), high-density lipoprotein (HDL) and HDL₂ cholesterol, in triglycerides, apolipoprotein B (apoB) and lipoprotein(a). Levels of HDL₃ cholesterol and apolipoprotein A1 (apoA1) were transiently decreased in the intranasal group. In the oral group, compared with the intranasal group, the decrease was larger for total and LDL cholesterol and lipoprotein(a), and smaller for triglycerides and apoA1. In the oral group, the ratios total/HDL cholesterol and LDL/HDL cholesterol were lowered, and of apoB/LDL cholesterol was increased, more than in the intranasal group.

**Conclusion(s):** Both intranasal and oral E₂/NET(A) therapy improved the lipid profile of healthy postmenopausal women, with some effects being more pronounced after oral administration.
Introduction

Postmenopausal hormone therapy (HT) carries both benefits and risks. Surrogate endpoint studies show that HT induces potentially beneficial changes in the lipid profile in postmenopausal women. Type and dosage of estrogen and progestogen used and the route and duration of administration affect the direction and the magnitude of the changes in the individual lipid parameters. Oral estrogen-alone therapy lowers total cholesterol (TC), low-density-lipoprotein cholesterol (LDL-C) and lipoprotein(a) [Lp(a)] and raises high-density-lipoprotein cholesterol (HDL-C) and triglycerides (TG).

Addition of a progestogen has little effect on the estrogen-induced reductions in TC and LDL-C, but can oppose the estrogen-induced increase in HDL-C and TG and might even lead to a decrease. The magnitude of these effects differs with the type and dosage of progestogen used. In contrast to oral administration, transdermal estradiol (E2) lowers TG, whereas the decrease in TC and LDL-C and the increase in HDL-C are diminished.

The traditional route of administration for HT is oral. However, intestinal and hepatic first-pass effects make high dosage necessary and lead to a variable biological availability. Furthermore, oral HT has been associated with an increased risk of venous thromboembolism. Transdermal estrogen administration, e.g. by patch, can prevent some of the problems associated with tablets, but absorption might still vary among women, and other disadvantages, such as adhesion problems and skin reactions can cause patient inconvenience. Recently, intranasal E2 administration (Aerodiol®, Servier, Courbevoie, France) has been shown to be a well-tolerated, effective, alternative route for E2 administration, avoiding the hepatic first-pass effect and resulting in a more reproducible exposure to E2.

Randomized studies have shown 300 μg intranasal E2 to be equally as effective as 2 mg oral E2 or a 50 μg releasing patch in relieving climacteric symptoms, but with fewer side effects. Intranasal E2 also induced changes in the lipid profile, which were comparable with transdermal E2 but smaller than those observed after oral administration. In nonhysterectomized women, intranasal E2 has to be combined with a separately administered progestogen (oral, intrauterine) for endometrial protection. To overcome the disadvantage of two modes of administration, a new intranasal spray was developed that combines E2 and norethisterone (NET) in one solution for continuous combined intranasal administration.

An international, randomized, double-blind, double-dummy study including 954 women was performed to compare the effects of the intranasal E2/NET formulation with those of oral low-dose continuous combined E2/NET acetate (NETA), a widely marketed combination. The primary endpoint was the effect on the endometrium. Among the secondary objectives in the original protocol was the effect on the lipid profile in Dutch participants; these results will be reported here.

Materials and methods

Participants

Healthy postmenopausal women aged 40 to 75 years were recruited from outpatient clinics and through advertisements in regional newspapers. All women were non-hysterectomized and had their
last menstrual period at least 2 years before inclusion. The postmenopausal state was confirmed hormonally (serum E$_2$ <110 pmol/L and follicle stimulating hormone (FSH) >30 mIU/mL). All participants had normal results on cervical smear and mammography within 12 months before inclusion. Normal results on transvaginal ultrasound and an endometrial biopsy without hyperplasia or polyps were required, as well as blood tests (lipids, liver enzymes, kidney function, glucose and thyroid stimulating hormone (TSH)) without any clinically relevant abnormalities. At screening all participants had to have plasma levels of total cholesterol ≤8.0 mmol/L and triglycerides of ≤3.0 mmol/L.

Exclusion criteria were a body-mass index (BMI) >32 kg/m$^2$, any contraindication for use of estrogen and/or progestogen, any ear-nose-throat disease that might interfere with intranasal drug administration, and concomitant use of the following treatments: any treatment for menopausal symptoms, chronic treatment liable to interfere with the coagulation profile, treatment liable to interfere with intranasal administration, enzyme inducers and systemic vasoconstrictors. Plasma lipids were analyzed in a subset of women participating in the Netherlands who had either no history of HT use, or had had a wash-out period of previous HT of at least 6 weeks before the baseline visit, and who were not taking lipid-lowering drugs.

All participants gave written informed consent before participation in the trial, which was conducted in accordance with the ethical principles stated in the Declaration of Helsinki, with Good Clinical Practice, and with approval of the central and local institutional review boards.

**Study design**

This study was performed in 13 centers in the Netherlands, included a total of 294 women, and was part of a large international, randomized, double-blind, double-dummy study with 2 parallel treatment arms including 954 women. After a 1 to 6-week screening period, eligible women were randomized to either one intranasal spray containing a fixed dose of 175 μg 17β-estradiol plus 275 μg NET (S21405, Institut de Recherches Internationales Servier (I.R.I.S.), Courbevoie, France) and one placebo capsule, or one capsule containing 1 mg 17β-estradiol plus 0.5 mg NETA (Activelle®, Novo Nordisk, Bagsvaerd, Denmark) and one placebo intranasal spray. Study medication was manufactured, packaged and labeled by I.R.I.S. (Courbevoie, France). Placebo and active treatments were identical in appearance and smell. Centralized computerized subject randomization was done by an Interactive Voice Response System (I.V.R.S.) in blocks of 12 (6 active intranasal spray and 6 active oral capsules) per center. Treatment was taken daily in the morning or in the evening at the same time for 52 weeks.

**Lipids and lipoprotein measurements**

For assessment of lipid parameters, venous blood samples were taken at baseline and in week 12, 24 and 52. Samples taken at the time of premature withdrawal of treatment were considered as samples taken during the next scheduled visit. After fasting and refraining from smoking for at least 10 hours and no alcohol intake for at least 24 hours, blood samples were taken between 8.00 and 10.00 a.m. After 20 minutes of rest, blood was collected into tubes containing K$_3$-EDTA (Becton Dickinson, Plymouth, United Kingdom). Within 1 hour after collection, plasma was separated by centrifugation at 1800 g and 20°C for 10 minutes. Plasma was divided into aliquots and stored at -80°C until shipment on dry ice to the central laboratory (Bio Analytical Research Corporation, BARC, Ghent, Belgium).
The following reagents were used: cholesterol esterase-oxidase (PAP) for TC, polyethylene glycol (PEG)-cholesterol esterase-oxidase (homogeneous) for HDL-C, PEG-precipitation (Quantolip) for HDL3-C, glycerophosphate oxidase-PAP for TG, and immunoturbidimetry for apolipoprotein B (apoB), apolipoprotein A1 (apoA1), and Lp(a). LDL-C was calculated using the Friedewald formula, and HDL3-C was computed from HDL-C and HDL3-C. All reagents were manufactured by Roche Diagnostics (Basel, Switzerland). All measurements were performed on a Roche Modular P800 (Roche Diagnostics, Basel, Switzerland). Overall coefficients of variation were as follows: 1.4-1.7% for TC, 2.1% for HDL-C, 6-12% for HDL3-C, 1.1-1.5% for TG, 5.4% for apoB, 3.1% for apoA1 and 2.7-4.2% for Lp(a).

Serum levels of E2 and of FSH were measured on the Roche E-170 Modular (Roche Diagnostics, Basel, Switzerland).

Throughout the whole study period, all participants, clinical investigators, and laboratory personnel were blinded as to the study medication. Unblinding was performed after all data were collected in the database.

Statistical analyses

Statistical analysis was performed with commercial software (Statistical Package for the Social Sciences 11.5 for Windows, SPSS Inc., Chicago, IL, USA). Values of lipids and lipoproteins are given as mean ± standard deviation or as median (25th to 75th percentile) if skewed. Percentage changes from baseline are given as mean (95% confidence interval [95%CI]), or as geometric mean (95%CI) if the changes had a skewed distribution.

Statistical analyses were performed with standard parametric tests; if the variables were skewed, analyses were done after log-transformation. Baseline values were compared using an unpaired t-test or a χ²-test where applicable. Within-group changes over time were tested using analysis of variance (ANOVA) for repeated measurements and paired t-tests versus baseline. For between-group comparisons we used analyses of covariance (ANCOVA) for repeated measurements, with the baseline value of the variable under consideration and age at baseline as constant covariates, and additional unpaired t-tests for percentage changes from baseline.

Results from women for whom data were available at baseline and at least at one other time point, were used for analyses; for ANOVA and ANCOVA for repeated measurements the last-observation-carried-forward procedure was applied for the missing values.

For Lp(a), our database showed many missing values because values were below the detection limit of the assay used (i.e. <60 mg/L). We performed analyses both on all available values and on values from the subgroup of women who had values available at all four measurement times (77 in the intranasal group and 52 in the oral). Results of the analyses were highly similar; the results of the subgroup analyses are given in Table I.

Results

Participants

Between September 2001 and June 2002, a total of 373 women were screened in the Netherlands, of whom 294 were randomized. Of these, 233 women were eligible for the current lipid substudy. Reasons for not being eligible were as follows: no wash-out from previous HT (n=49), use of lipid-
lowering drugs (n=4), no baseline blood samples (n=3), no start of study medication (n=3), or less than 2 years post-menopausal (n=2) (Figure 1). Baseline characteristics of the two groups did not differ (Table I), except for age. Importantly, menopausal age did not differ between the two groups. Because of this difference in age, we used age as an additional covariate in the ANCOVA. This did not substantially affect the results; results of analyses with both baseline and age as covariate are shown.

During the study, 37 women discontinued study medication: 11 in the intranasal versus 26 in the oral group (P<0.01) (Figure 1). The main reason for premature study discontinuation was the occurrence of an adverse event. No women stopped because of the occurrence of coronary heart disease (CHD) or cerebrovascular disease. In the intranasal group, two women stopped because of the occurrence of venous thrombosis; in the oral group, one woman discontinued because of clinical symptoms fitting a venous thrombosis which, however, could not be confirmed ultrasonographically, and one woman stopped because of the detection of breast cancer. More women in the oral than the intranasal group stopped because of mastalgia (3 versus 0), vaginal bleeding (5 versus 2) or headache/migraine (4 versus 0). Statistical analyses were based on 222 women (112 in the intranasal and 110 in the oral group) for whom values at baseline and at least at one other measurement point were available. Baseline characteristics of the women included in the analyses did not differ from all randomized women (n=294) except for mean E2 level, which was higher in the latter group as most women were not eligible for the substudy because of recent HT use. The last participants completed the study in May 2003.

### Table I. Baseline characteristics

<table>
<thead>
<tr>
<th></th>
<th>Intranasal</th>
<th>Oral</th>
<th>P-valuea</th>
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<tbody>
<tr>
<td>n</td>
<td>116</td>
<td>117</td>
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</tr>
<tr>
<td>Age (yr)</td>
<td>56.8 ± 5.6</td>
<td>54.9 ± 4.5</td>
<td>&lt;0.01</td>
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<tr>
<td>Amenorrhoea (months)</td>
<td>70 (44-115)</td>
<td>63 (36-105)</td>
<td>0.24</td>
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<tr>
<td>Weight (kg)</td>
<td>69.5 ± 9.8</td>
<td>70.0 ± 9.9</td>
<td>0.70</td>
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<tr>
<td>Body mass index (kg/m²)</td>
<td>25.2 ± 3.1</td>
<td>25.4 ± 3.3</td>
<td>0.57</td>
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<tr>
<td>Blood pressure (mmHg)</td>
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<tr>
<td>systolic</td>
<td>127 ± 16</td>
<td>130 ± 19</td>
<td>0.31</td>
</tr>
<tr>
<td>diastolic</td>
<td>81 ± 10</td>
<td>80 ± 11</td>
<td>0.62</td>
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<tr>
<td>Waist/hip-ratio</td>
<td>0.81 ± 0.06</td>
<td>0.81 ± 0.06</td>
<td>0.89</td>
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<tr>
<td>Smoking, n (%)</td>
<td>27 (23.3%)</td>
<td>28 (23.9%)</td>
<td>0.91</td>
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<tr>
<td>Serum E₂ (pmol/L)</td>
<td>68 (48-85)</td>
<td>65 (50-80)</td>
<td>0.93</td>
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<tr>
<td>Serum FSH (IU/L)</td>
<td>79 ± 29</td>
<td>85 ± 29</td>
<td>0.13</td>
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</tbody>
</table>

Values are given as mean ± SD, as median (25th – 75th percentile), or n(%).

Intranasal, spray containing 175 μg E₂ and 275 μg norethisterone; oral, capsule containing 1 mg E₂ and 0.5 mg norethisterone acetate

P-value for t-test (for amenorrhoea and oestradiol after log-transformation) or χ²-test for between-group differences.
Lipids and lipoproteins

Baseline values of lipids and lipoproteins were similar between the groups (Table II). Within both groups we found a significant reduction in all lipids and lipoproteins, except for apoA1 in the oral group (Table II, Figure 2). With respect to TC, LDL-C, HDL-C, HDL2-C, apoB and Lp(a) in both groups, and for TG in the intranasal group, the reductions were already present in week 12 and persisted during the following 40 weeks. In the oral group the reduction in TG reached statistical significance in week 52; and for HDL3-C en apoA1 the reductions were transiently significant in the first 24 weeks of treatment.

Decreases in the oral group were significantly larger, compared with the intranasal group, for TC, LDL-C and Lp(a) and smaller for TG and apoA1. No between-group differences were observed for the changes in HDL-C, HDL2-C, HDL3-C and apoB.

Figure 1. Trial profile

n, number of subjects.
intranasal, spray containing 175 μg E2 and 275 μg norethisterone; oral, capsule containing 1 mg E2 and 0.5 mg norethisterone acetate.
Evaluable subjects at week 12, 24 and 52: 112, 110 and 108 in the intranasal group, and 110, 103 and 95 in the oral group, respectively.
### Table II. Lipids and lipoproteins

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Week 12</th>
<th>Week 24</th>
<th>Week 52</th>
<th>% Δ 0-52</th>
<th>P (ANCOVA)</th>
<th>P (ANOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TC</strong></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Intranasal</td>
<td>5.96 ± 0.97</td>
<td>5.48 ± 0.91&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.42 ± 0.92&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.42 ± 0.94&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-8.5 (-10.5 to -6.5)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Oral</td>
<td>6.02 ± 0.90</td>
<td>5.43 ± 0.84&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.30 ± 0.83&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.36 ± 0.87&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-11.1 (-13.3 to -8.9)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<tr>
<td>P&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.66</td>
<td></td>
<td></td>
<td></td>
<td>0.09</td>
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<tr>
<td><strong>LDL-C</strong></td>
<td></td>
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</tr>
<tr>
<td>Intranasal</td>
<td>3.81 ± 0.93</td>
<td>3.52 ± 0.85&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.50 ± 0.90&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.45 ± 0.89&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-8.5 (-11.2 to -5.8)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Oral</td>
<td>3.82 ± 0.83</td>
<td>3.37 ± 0.85&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.28 ± 0.81&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.34 ± 0.87&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-13.0 (-16.1 to -10.0)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<tr>
<td>P&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.96</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.05</td>
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<tr>
<td><strong>HDL-C</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.48</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intranasal</td>
<td>1.55 ± 0.39</td>
<td>1.44 ± 0.36&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.43 ± 0.32&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.46 ± 0.31&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-4.3 (-7.1 to -1.4)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<tr>
<td>Oral</td>
<td>1.63 ± 0.46</td>
<td>1.54 ± 0.38&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.49 ± 0.38&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.53 ± 0.35&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-3.5 (-7.2 to 0.1)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<tr>
<td>P&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.16</td>
<td></td>
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<td></td>
<td>0.75</td>
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<tr>
<td><strong>HDL&lt;sub&gt;2&lt;/sub&gt;-C</strong></td>
<td>0.43 (0.31-0.62)</td>
<td>0.41 (0.29-0.52)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.36 (0.30-0.49)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.36 (0.26-0.44)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-19.7 (-24.8 to -14.2)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Intranasal</td>
<td>0.47 (0.34-0.65)</td>
<td>0.41 (0.31-0.49)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.41 (0.29-0.52)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.36 (0.29-0.49)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-20.5 (-27.0 to -13.5)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<tr>
<td>Oral</td>
<td>0.26</td>
<td></td>
<td></td>
<td></td>
<td>0.85</td>
<td></td>
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<tr>
<td><strong>HDL&lt;sub&gt;3&lt;/sub&gt;-C</strong></td>
<td>1.08 ± 0.24</td>
<td>1.02 ± 0.23&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.03 ± 0.23&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.09 ± 0.22&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.2 (-1.1 to 5.5)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<tr>
<td>Intranasal</td>
<td>1.12 ± 0.27</td>
<td>1.10 ± 0.25&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.07 ± 0.23&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.14 ± 0.23&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.9 (0.5 to 9.3)</td>
<td>&lt;0.01</td>
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<tr>
<td>Oral</td>
<td>0.28</td>
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<td>0.33</td>
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<tr>
<td><strong>TG</strong></td>
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<td></td>
<td>0.14</td>
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<tr>
<td>Intranasal</td>
<td>1.20 (0.94-1.56)</td>
<td>1.10 (0.82-1.25)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.01 (0.82-1.30)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.06 (0.81-1.32)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-11.7 (-16.8 to -6.3)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Oral</td>
<td>0.99 (0.79-1.45)</td>
<td>1.01 (0.78-1.31)</td>
<td>1.03 (0.79-1.33)</td>
<td>0.96 (0.75-1.30)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>-8.9 (-14.5 to -2.9)</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
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<tr>
<td>P&lt;sup&gt;d&lt;/sup&gt;</td>
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<td></td>
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<td>0.47</td>
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<tr>
<td><strong>ApoB</strong></td>
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<td></td>
<td>0.88</td>
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<tr>
<td>Intranasal</td>
<td>116 ± 22</td>
<td>109 ± 23&lt;sup&gt;c&lt;/sup&gt;</td>
<td>106 ± 24&lt;sup&gt;c&lt;/sup&gt;</td>
<td>108 ± 25&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-7.0 (-9.2 to -4.7)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<tr>
<td>Oral</td>
<td>115 ± 21</td>
<td>110 ± 23&lt;sup&gt;c&lt;/sup&gt;</td>
<td>105 ± 24&lt;sup&gt;c&lt;/sup&gt;</td>
<td>107 ± 25&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-7.4 (-10.1 to -4.6)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>P&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.76</td>
<td></td>
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<tr>
<td><strong>ApoA1</strong></td>
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<td>&lt;0.001</td>
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<tr>
<td>Intranasal</td>
<td>143 ± 23</td>
<td>134 ± 22&lt;sup&gt;c&lt;/sup&gt;</td>
<td>138 ± 23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>140 ± 22&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-1.9 (-5.1 to 1.4)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Oral</td>
<td>148 ± 26</td>
<td>149 ± 24</td>
<td>148 ± 27</td>
<td>149 ± 24</td>
<td>0.9 (-1.9 to 3.7)</td>
<td>0.98</td>
<td>0.21</td>
</tr>
<tr>
<td>P&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.09</td>
<td></td>
<td></td>
<td></td>
<td>0.21</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Lp(a)&lt;sup&gt;9&lt;/sup&gt;</strong></td>
<td>320 (185-655)</td>
<td>300 (160-595)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>290 (140-515)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>300 (145-560)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-15.7 (-20.8 to -10.3)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Oral</td>
<td>440 (230-848)</td>
<td>360 (125-585)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>370 (145-568)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>375 (153-625)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-24.3 (-28.5 to -19.9)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>P&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.37</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.05</td>
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</tbody>
</table>
Values at baseline and at week 12, 24 and 52 as mean ± SD or median (25th – 75th percentile); %Δ 0-52, percentage change from baseline in week 52 given as (geometric) mean (95% CI). Units are in mmol/L for TC, LDL-C, HDL-C and TG; in mg/100mL for apoB and apoA1; and in mg/L for Lp(a).

TC, total cholesterol; LDL, low-density lipoprotein; HDL, high-density lipoprotein; TG, triglycerides; apoB, apolipoprotein B; apoA1, apolipoprotein A1 and Lp(a); intranasal, spray containing 175 μg 17β-oestradiol (E2) and 275 μg norethisterone; oral, capsule containing 1 mg E2 and 0.5 mg norethisterone acetate

aAnalysis of covariance for repeated measurements over 52 weeks with the baseline value of the parameter under consideration and age at baseline as covariates for between-group differences in change
bAnalysis of variance for repeated measurements for within-group changes over the 52 weeks

% change from baseline: *P<0.05, #P<0.01 and §P<0.001
Difference in percentage change between the groups: †P<0.05, ††P<0.01 and ‡P<0.001
Atherogenic indices

Within the oral group we found a significant reduction in the TC/HDL-C and LDL-C/HDL-C ratios and an increase in the apoB/LDL-C ratio. These changes were already present in week 12 and sustained in weeks 24 and 52 (Table III).

Within the intranasal group a reduction in the TC/HDL-C ratio was found. The changes in the LDL-C/HDL-C and apoB/LDL-C ratios did not reach statistical significance.

The decrease in the TC/HDL-C and LDL-C/HDL-C ratios and the increase in the apoB/LDL-C ratio were significantly larger in the oral compared to the intranasal group.

**Table III. Atherogenic indices**

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Week 12</th>
<th>Week 24</th>
<th>Week 52</th>
<th>% ∆ 0-52</th>
<th>P  (ANCOVA)</th>
<th>P  (ANOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TC/HDL-C</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intranasal</td>
<td>3.83 (3.13-4.82)</td>
<td>3.92 (3.13-4.59)</td>
<td>3.83 (3.10-4.58)</td>
<td>3.87 (2.99-4.47)</td>
<td>-3.9 (-6.7 to -1.1)</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Oral</td>
<td>3.64 (3.02-4.66)</td>
<td>3.50 (2.86-4.46)</td>
<td>3.51 (2.92-4.41)</td>
<td>3.31 (2.85-4.38)</td>
<td>-6.4 (-9.4 to -3.3)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>P&lt;</td>
<td>0.40</td>
<td></td>
<td></td>
<td></td>
<td>0.23</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>LDL-C/HDL-C</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intranasal</td>
<td>2.43 (1.89-3.32)</td>
<td>2.57 (1.87-3.15)</td>
<td>2.46 (1.84-3.16)</td>
<td>2.52 (1.73-3.01)</td>
<td>-4.5 (-8.3 to -0.6)</td>
<td>&lt;0.001</td>
<td>0.10</td>
</tr>
<tr>
<td>Oral</td>
<td>2.32 (1.78-3.18)</td>
<td>2.14 (1.63-3.00)</td>
<td>2.17 (1.75-2.91)</td>
<td>2.04 (1.60-2.91)</td>
<td>-9.1 (-13.1 to -4.9)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>P&lt;</td>
<td>0.51</td>
<td></td>
<td></td>
<td></td>
<td>0.11</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ApoB/LDL-C</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intranasal</td>
<td>31.1 ± 3.1</td>
<td>31.5 ± 3.4</td>
<td>30.7 ± 4.1</td>
<td>31.7 ± 2.4</td>
<td>2.5 (0.8 to 4.2)</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>Oral</td>
<td>30.6 ± 2.8</td>
<td>33.0 ± 3.3</td>
<td>32.2 ± 3.8</td>
<td>32.5 ± 2.7</td>
<td>7.1 (4.7 to 9.4)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>P&lt;</td>
<td>0.31</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.01</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Ratios at baseline and at week 12, 24 and 52 as mean ± SD or median (25th - 75th percentile); %Δ 0-52, percentage change from baseline in week 52 given as (geometric) mean (95% confidence interval). TC, total cholesterol; HDL-C, high-density lipoprotein; LDL-C, low-density lipoprotein; apoB, apolipoprotein B; intranasal, spray containing 175 μg 17β-oestradiol (E2) and 275 μg norethisterone; oral, capsule containing 1 mg E2 and 0.5 mg norethisterone acetate.

*a*Analysis of covariance for repeated measurements over 52 weeks with the baseline value of the parameter under consideration and age at baseline as covariates for between-group differences in change, *b*Analysis of variance for repeated measurements for within-group changes over the 52 weeks, *c*P<0.01 versus baseline (paired t-test), *d*P<0.001 versus baseline (paired t-test), *e*unpaired t-test for comparison between the groups, *f*P<0.05 versus baseline (paired t-test).

Discussion

In this study, we investigated the effect of a new route of administration of combined estrogen plus progestogen therapy. In contrast to the prolonged estrogen exposure after oral and transdermal administration, intranasally administered E2 is rapidly absorbed and induces very steep and short peaks in plasma levels. This short-term, pulsed exposure to E2 induces long-term effects by activating
the nuclear estrogen receptor, thus initiating the signal transduction cascade. This results in gene activation that ultimately induces a specific biological event.11

Some target tissues appear to be more sensitive to pulsed estrogen exposure than others. An important potential advantage of this mechanism is less stimulation of the breast and endometrium.12 In our study fewer women in the intranasal group compared to the oral group discontinued the study because of adverse events. The observed lower number of women with breakthrough bleeding and mastalgia in the intranasal group was expected in line with a previous study.7

Intranasal administration, compared with oral, requires a lower dose because of its high bioavailability and induces less variability in bioavailability for both E2 and NETA (unpublished data). Previous studies showed 300 μg intranasal E2 to be equivalent to 2 mg oral E2 in clinical efficacy.7 The 24-hour exposure after 2 mg oral E2 or 50 μg transdermal E2 was similar to 300 μg intranasal E2.13 In line with the current recommendation to use low dose HT, half dose of E2 was administered in this study. The prodrug NETA is rapidly hydrolyzed into the active hormone NET. Equivalent quantities of NETA and NET provide similar pharmacokinetic profiles for NET concentrations (unpublished data). NET was used in the intranasal spray because it is the active hormone and is easy to solute.

In this 1-year, randomized, double-blind study, we found a potentially beneficial decrease from baseline in TC, LDL-C, TG, apoB and Lp(a) during both intranasal E2/NET and oral E2/NETA therapy and an undesirable decrease in HDL-C, HDL2-C, HDL3-C in both groups and in apoA1 in the intranasal group. In the oral group, the changes were significantly larger in TC, LDL-C and Lp(a) and significantly smaller in TG and apoA1 compared with the changes in the intranasal treatment group. The atherogenic indices TC/HDL-C and LDL-C/HDL-C were lowered and apoA1/LDL-C was raised more in the oral than the intranasal group.

The decrease in TC, LDL-C, HDL-C, TG and Lp(a) in the oral E2/NETA group is in line with the meta-analysis by Godsland2 that reported similar results for E2 continuous combined with NETA as a net effect of 17 studies. Norethisterone is a 19-nortestosterone derivative with a partial androgenic activity, which can reverse estrogen-induced effects independent of its route of administration.3 In a direct comparison, NETA induced an inversion of the oral E2-induced increase in TG and HDL-C into a decrease, and an augmentation of the reduction in Lp(a) levels.14

Until now, only one study has investigated the effect of intranasal E2 on the lipid profile.7 After 24 weeks of intranasal E2 sequentially combined with oral dydrogesterone, TC, apoB and Lp(a) were significantly decreased. Oral E2 sequentially combined with oral dydrogesterone, the comparison treatment group in that study, induced a significantly larger decrease in LDL-C and a significantly larger increase in HDL-C and TG compared with intranasal administration.

Transdermal administration is another non-oral route for HT. The metabolic effects of transdermal therapy are likely to be comparable with intranasal therapy with respect to bypassing the hepatic first-pass effect. Transdermal E2 is known to raise HDL-C and to lower TC, LDL-C and also TG. For (mainly sequentially) combined transdermal E2 plus transdermal NETA regimens, Godsland reported an overall nearly neutral effect on HDL-C and decreases comparable with unopposed transdermal E2 in TC, LDL-C and TG, as well as a decrease in Lp(a).2 Recently, Stevenson et al.15 reported that 6-month continuous combined transdermal E2 50 μg/NETA 125 μg significantly decreased TC, HDL-C, HDL3-C and TG, but had no significant effects on LDL-C, HDL2-C, apoA1, apoB, or Lp(a).

The lowering of LDL-C levels by oral estrogens is the result of an increased clearance through a higher number and activity of LDL receptors. The larger decrease in LDL-C in the oral group suggests
a more favorable effect in this group. However, the concomitant decrease in both groups in apoB led to a significant increase in apoB/LDL-C ratio in the oral group, which was not observed in the intranasal group. This increase reflects an increase in LDL density and a decrease in particle size, which is associated with coronary atherosclerosis.\textsuperscript{17} A shift from intermediate towards a smaller LDL particle size after oral HT has been reported earlier.\textsuperscript{18} Others showed that this decrease in mean particle size was due to a decrease of concentration in large LDL rather than an increase in small LDL.\textsuperscript{19}

Lipoprotein(a) is regarded as an independent risk factor for the development of coronary heart disease.\textsuperscript{20} It contains apoB\textsubscript{100}, like LDL, which is responsible for its atherogenicity, but also an apolipoprotein(a) [apo(a)] molecule that is considered responsible for its thrombogenic properties.\textsuperscript{21} Lipoprotein(a) levels can be lowered by estrogens by an increased clearance; the hepatic LDL receptor binds to the apoB\textsubscript{100} component, although this is partially covered by the apo(a) molecule. Another mechanism is a reduced synthesis of apo(a).\textsuperscript{22}

The larger effect on TG and apoA1 observed in the intranasal group might be mainly NET induced. Independent of its route of administration, NET can lower both TG and HDL-C in such extent that it can even completely counteract the E\textsubscript{2}-induced effects.\textsuperscript{3} When the TG-lowering effect of NET is added to the intranasal E\textsubscript{2}-induced decrease or the oral E\textsubscript{2}-induced increase, this could result in a larger reduction in TG in the intranasal than the oral group. Compared with the oral group, the potentially unfavorable larger reduction in apoA1 in the intranasal group appears to be reflected in a larger reduction in HDL-C and the HDL\textsubscript{3}-C subfraction; this difference, however, is small and not statistically significant.

Lowering plasma levels of total and LDL-C by statins is known to improve the cardiovascular outcome in secondary prevention\textsuperscript{23} as well as in primary prevention in normocholesterolemic men and women.\textsuperscript{24} The clinical relevance of obtaining improvement in the lipid profile under HT remains unclear. Observational studies have reported a 50% reduction in the risk for CHD by postmenopausal HT,\textsuperscript{25} a clinical effect which is in line with many randomized surrogate endpoint studies reporting an improvement in the lipid profile,\textsuperscript{2} as well as in other markers, such as homocysteine and endothelial function.

More recently, in the Heart and Estrogen/progestin Replacement Study (HERS)\textsuperscript{26} and the Women's Health Initiative (WHI) trial,\textsuperscript{27} conjugated equine estrogens plus medroxyprogesterone acetate induced favorable lipid profile changes without reducing the risk of CHD in predominately late postmenopausal women who started HT 10-15 years after menopause. Moreover, the women who participated in the HERS and WHI trial were older than the women to whom HT normally is prescribed in clinical practice.

Atherosclerosis is common at higher age and is likely to impair the endothelial effects induced by HT. Thus, the negative HERS and WHI results do not per se exclude antiatherogenic effects of HT in younger, healthier postmenopausal women. Indeed, in healthy women starting HT early after menopause, a cardioprotective effect has been suggested,\textsuperscript{28,29} but this has yet to be established. The lack of a cardioprotective effect despite improvements in the lipid profile might also be due to simultaneously induced unfavorable effects on other risk markers. Oral HT has shown to negatively influence the procoagulant / anticoagulant balance\textsuperscript{30} and to increase serum C-reactive protein levels.\textsuperscript{31} These unfavorable effects are much smaller after transdermal E\textsubscript{2} administration.\textsuperscript{32,33}

Among the strengths of our study are the randomized, double-blind study design, the large number of participants and the 1-year duration. The study also has limitations. We did not include an
untreated or placebo control group. However, we compared the effects of a new product with a well studied reference product, and the effects we found for this product were comparable with previous placebo-controlled studies. Second, at baseline the groups differed in age. Therefore we added age as a covariate in the analysis of covariance; however, this did not influence our results, and furthermore, the groups were comparable with respect to time since menopause. Third, we used a relatively short washout period from previous HT of 6 weeks. In line with the findings by Koh et al. this might be considered sufficient to eliminate effects of previous HT. Re-analysis of our data without the 24 women having a washout of 6 to 12 weeks revealed similar results (data not shown).

In conclusion, in this study in healthy postmenopausal women, intranasal continuous combined E₂/NET therapy showed potentially favorable effects on the lipid profile, which were, to some extent, smaller than those observed during oral E₂/NETA therapy.

Acknowledgements

We thank all the women who participated in the study; the following (co-)investigators in the participating centers: DH Bogchelman, MD, Groningen; GL Bremer, MD, PhD, Eindhoven; DDM Braat, MD, PhD, and C Klipping, MD, Nijmegen; JM Burggraaff, MD, Emmen; HR Franke, MD, PhD, Enschede; S Friese, MD, PhD, Breda; J de Graaff, MD, PhD, Tilburg; J Kropveld, MD, Hengelo; AMM Pennings, MD, and PWA Houben, MD, PhD, Den Helder; HS The, MD, PhD, Nieuwegein; TE Vogelvang, MD, and MO Verhoeven, MD, Amsterdam; and the center’s research staff.

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Chapter 5


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No increase in C-reactive protein levels during intranasal compared to oral hormone therapy in healthy postmenopausal women

Majoie Hemelaar
Peter Kenemans
Casper G Schalkwijk
Didi DM Braat
Marius J van der Mooren


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Abstract

**Background:** Inflammation plays an important role in the development of atherosclerotic disease. Oral postmenopausal hormone therapy increases serum C-reactive protein (CRP) levels. This study compared the effects of intranasal and oral administration of 17β-estradiol (E₂) combined with norethisterone (acetate) [NET(A)] on markers of inflammation in healthy postmenopausal women.

**Methods:** Ninety healthy postmenopausal women (age 56.6 ± 4.7 years) participated in this 1-year trial. After computerized block randomisation, they daily received, in a double-blind fashion, either intranasal E₂/NET 175 μg/275 μg (n=47) or oral E₂/NETA 1 mg/0.5 mg (n=43). Concentrations of high sensitivity CRP and adhesion molecules were measured at baseline and after 12, 24 and 52 weeks of treatment.

**Results:** CRP levels were increased (P=0.001) in the oral but not in the intranasal group. The increase in the oral group was highest in week 12 (64.9%) and was larger (P<0.01) compared with the non-significant increase (8.6%) found in the intranasal group. Both groups showed decreases (P<0.001) in soluble vascular cell adhesion molecule (sVCAM), soluble intercellular adhesion molecule (sICAM) and sE-selectin. The decreases were larger (P<0.01) in the oral than in the intranasal group.

**Conclusion:** Intranasal E₂/NET therapy did not significantly increase CRP levels, in contrast to the increase observed in the oral E₂/NETA treatment group. Both intranasal and oral therapy lowered plasma concentrations of adhesion molecules, however, more so in the oral group.
Introduction

The evidence available as to the effect of postmenopausal hormone therapy (HT) on cardiovascular risk is conflicting. In contrast to observational studies reporting a risk reduction among HT users,1 randomized controlled trials reported no effect or even an early increased risk for the development of coronary heart disease (CHD) among women assigned to oral HT.2-4

Inflammation plays an important role in the process of atherosclerosis and plaque formation.5 Acute phase proteins, especially C-reactive protein (CRP), play a role in non-specific defence mechanisms. High sensitive CRP (hsCRP) has been shown to be a predictor of future cardiovascular events.6,7 In response to endothelial dysfunction, cell adhesion molecules are expressed on endothelial cells to attract leukocytes. Elevated plasma levels of these molecules have been associated with an increased CHD risk.8,9 Most controlled studies report an increase in CRP levels during oral HT.10-14 During transdermal HT the liver, the major production site of CRP, is bypassed. Most studies found no increase during transdermal HT.11,14-18 For adhesion molecules, both oral and transdermal HT studies have shown inconsistent results.15,19,20

In addition to the oral and transdermal route of administration, a spray has become available for intranasal administration of estradiol (E2) (Aerodiol®, Servier, Courbevoie, France). The intranasal route has been shown to be a well-tolerated, effective alternative.21-23 Less intra- and inter-subject variability was observed in E2 exposure.21,22 As a successor to the E2-only spray, an intranasal spray for continuous combined 17β-estradiol and norethisterone (E2/NET) administration has been developed. As hepatic metabolism is largely bypassed, it is plausible that, just as transdermal patches or gels and the intranasal E2-only spray24 have little effect on CRP levels, the same may be true of the intranasal E2/NET combined spray. In the present study, the effects on CRP and adhesion molecules of the intranasal E2/NET formulation were compared with those of oral low-dose continuous combined E2/NETA, a widely marketed combination. Nested within a large international, randomized, double-blind, double-dummy study, which had endometrial safety as the primary endpoint, effects on CRP and adhesion molecules were studied as a secondary objective among participants in two Dutch centres.

Materials and methods

Participants

Healthy postmenopausal women, aged 40 to 75 years, were recruited from outpatient clinics and through advertisements in regional newspapers. All women were non-hysterectomized and had their last menstrual period at least 2 years before inclusion. Serum E2 had to be <30 pg/mL with FSH >30 mIU/mL. All participants had a normal cervical smear and mammography within 12 months before inclusion. A normal transvaginal ultrasound and an endometrial biopsy without hyperplasia or polyps were required, as well as blood tests (lipids, liver enzymes, kidney function, glucose and thyroid-stimulating hormone) without any clinically relevant abnormalities. At screening all participants had plasma levels of total cholesterol of ≤8.0 mmol/L and of triglycerides of ≤3.0 mmol/L. Exclusion criteria were a BMI >32 kg/m², any contra-indication to the use of estrogen and/or progestogen, any ear-nose-throat disease that might interfere with intranasal drug administration, and concomitant use of the following treatments: any treatment for menopausal symptoms, chronic
treatment liable to interfere with the coagulation profile, treatment liable to interfere with intranasal administration, enzyme inducers, and systemic vasoconstrictors. Inflammation parameters were measured in a subset of women who either had no history of use of HT or had a wash-out of previous HT of at least 6 weeks before the baseline visit and who were not taking lipid-lowering drugs.

All participants gave written informed consent before inclusion in the trial, which was conducted in accordance with the ethical principles stated in the Declaration of Helsinki, with Good Clinical Practice, and with approval of the central and local institutional review boards.

**Study design**

This study was performed in 90 women who were included in two centres in the Netherlands, as part of a large international, randomized, double-blind, double-dummy study with two parallel treatment arms including 954 women in total. After a 1 to 6 weeks screening period, eligible women were randomized to either one intranasal spray containing a fixed dose of 175 μg 17β-estradiol + 275 μg norethisterone (S21405, Servier, Courbevoie, France) and one placebo capsule (intranasal E2/NET group) or one capsule containing 1 mg 17β-estradiol + 0.5 mg norethisterone acetate (Activelle®, Novo Nordisk, Bagsvaerd, Denmark) and one placebo intranasal spray (oral E2/NET group) daily. Study medication was manufactured, packaged and labelled by the Institut de Recherches Internationales Servier (I.R.I.S.; Courbevoie, France). Placebos and active treatments were identical in appearance and smell. Centralized computerized subject randomisation was done by an Interactive Voice Response System (I.V.R.S.) in blocks of 12 (six active spray and six active capsules) per centre. Treatment was administered for 52 weeks.

Throughout the whole study period, all participants, clinical investigators and laboratory personnel were blinded for the study medication. Unblinding was done after all data were collected in the database.

**Markers of inflammation**

To assess concentrations of CRP and adhesion molecules, venous blood samples were taken at baseline and in week 12, 24 and 52. Blood samples were taken between 8.00 and 10.00 a.m. after fasting and non-smoking for at least 10 hours and no alcohol intake for at least 24 hours. After 20 minutes of rest, blood was collected into plain tubes at room temperature (Becton Dickinson, Plymouth, UK) for CRP or into pre-cooled tubes containing K3-EDTA for soluble vascular cell adhesion molecule (sVCAM), soluble intercellular adhesion molecule (sICAM) and sE-selectin. After blood collection, pre-cooled tubes were immediately put back into ice. Within 1 hour after collection, plasma was separated by centrifugation at 2000 g for 30 minutes at 20°C (for blood collected into plain tubes) or at 4°C (for blood collected into cooled tubes containing K3-EDTA). Plasma was divided into aliquots, snap-frozen and stored at -80°C until analysis.

High sensitivity CRP was assayed by an in-house sensitive ELISA with a lower limit of detection of 0.01 mg/L. Adhesion molecules were determined by commercially available ELISAs (Elipair, Diaclone, Besançon Cedex, France). The intra- and inter assay coefficients of variation (CVs) were 3.9% and 8.7% for CRP, 4.4% and 5.1% for sVCAM, 4.0% and 6.4% for sICAM, and 4% and 10% for sE-selectin, respectively. All samples of a given subject were assayed within a single run.

Plasma levels of E2 and of FSH were measured on the Roche E-170 Modular (Roche, Basel, Switzerland) using electrochemiluminescence immunoassay (ECLIA), serum sex-hormone binding
globulin (SHBG) levels were measured using an immunoradiometric assay (IRMA) (Orion Diagnostica, Espoo, Finland) which has an overall CV of 10%.

**Statistical analyses**

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) 11.5 for Windows (SPSS Inc., Chicago, IL, USA). Concentrations of the inflammation parameters are given as mean ± standard deviation or as median (25th to 75th percentile) if skewed. Percentage changes from baseline are given as mean [95% confidence interval (95%CI)] or as geometric mean (95%CI) if the changes had a skewed distribution.

Statistical analyses were performed using standard parametric tests; if the variables were skewed, analyses were done after log-transformation. Baseline values were compared using an unpaired t-test or a χ²-test where applicable. Within-group changes over time were tested using analysis of variance (ANOVA) for repeated measurements and paired t-tests versus baseline. For between-group comparisons we used analyses of covariance (ANCOVA) for repeated measurements, with the baseline value of the variable under consideration as constant covariate. Percentage changes from baseline where compared with unpaired t-tests.

Only data from women, for whom data were available at baseline and at least at one other time point, were used for the analyses; for ANOVA and ANCOVA for repeated measurements the last-observation-carried-forward procedure for the missing values was applied.

As CRP levels are raised by inflammatory conditions, we re-analysed CRP data after exclusion of women (n=10) who reported an infection in the weeks prior to blood sampling or those with a CRP concentration ≥ 10 mg/L, which is considered as the lower threshold level for the existence of an acute inflammation.

Correlations for baseline values and absolute changes in CRP and adhesion molecules were analysed by calculation of the Pearson's correlation coefficient. Correlations with changes in SHBG plasma levels were analysed in the subset of women of whom SHBG values were available (n=65, 54 and 51 at week 12, 24 and 52, respectively).

Sample size calculation for this cardiovascular substudy was based on changes in normalised APC sensitivity ratio (nAPCsr), a strong haemostatic risk factor associated with an increased venous thrombotic risk, and is the pathophysiologic mechanism behind the Factor V Leiden mutation, a hereditary risk factor for venous thrombosis. To find a 35% difference in change in nAPCsr between the groups with a standard deviation in percentage change of 75%, using a power of 80% and an α of 5% (two-sided), 37 evaluable women would be required in each group.

**Results**

**Participants**

Between September 2001 and June 2002, 125 women were screened in two participating centres, of whom 94 women were randomised. As four women either had no wash-out from previous HT (n=3) or used lipid-lowering drugs (n=1), 90 women were found to be eligible for the current substudy (Figure 1). The last group of patients completed the study in May 2003. Baseline demographic characteristics did not differ between the two treatment groups (Table I).
Two women in the intranasal group discontinued the study compared with 10 in the oral group (P<0.01) (Figure 1). Premature study discontinuation was mainly related to the occurrence of an adverse event. No women stopped because of the occurrence of a coronary or a cerebrovascular event. One woman in the intranasal group discontinued in week 11 because of the occurrence of a deep venous thrombosis which, in retrospect, probably was already present before study entry. Another woman, in the oral group, discontinued in week 36 because of clinical symptoms fitting a venous thrombosis which, however, could not be confirmed ultrasonographically. Furthermore, one woman in the oral group stopped because of the detection of breast cancer in week 38 and one woman in the intranasal group was excluded from analyses after week 12 because of the start of preventive anticoagulant therapy on account of a family history of cerebrovascular disease.

Analyses were therefore based on 86 women (46 in the intranasal and 40 in the oral group) for whom values at baseline and at least at one other time point were available.

### Markers of inflammation

Baseline values of CRP and adhesion molecules were similar between the groups (Table II).

During the 52-week study period, CRP levels increased significantly (P=0.001) in the oral, but not in the intranasal group (Table II, Figure 2a). The difference between the groups was statistically significant (P=0.01). At week 12 and 24, the increases from baseline in the oral group were 64.9% (P=0.001) and 49.1% (P<0.01), respectively, whereas non-significant increases of 8.6% and 29.8% were found in the intranasal group. The difference between the groups in week 12 was statistically

<table>
<thead>
<tr>
<th>Table I. Baseline characteristics</th>
<th>Intranasal</th>
<th>Oral</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>47</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>Age (yr)</td>
<td>57.5 ± 5.2</td>
<td>55.9 ± 3.9</td>
<td>0.11</td>
</tr>
<tr>
<td>Amenorrhea (months)</td>
<td>74 (51-123)</td>
<td>73 (45-106)</td>
<td>0.86</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>68.4 ± 9.2</td>
<td>68.5 ± 9.3</td>
<td>0.96</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>24.8 ± 3.3</td>
<td>25.2 ± 3.6</td>
<td>0.56</td>
</tr>
<tr>
<td>Blood pressure (mmHg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>systolic</td>
<td>126 ± 17</td>
<td>121 ± 17</td>
<td>0.19</td>
</tr>
<tr>
<td>diastolic</td>
<td>80 ± 10</td>
<td>76 ± 11</td>
<td>0.08</td>
</tr>
<tr>
<td>Smoking n (%)</td>
<td>12 (27.7%)</td>
<td>11 (25.6%)</td>
<td>0.82</td>
</tr>
<tr>
<td>Serum cholesterol (mmol/L)</td>
<td>6.1 ± 1.1</td>
<td>6.1 ± 0.8</td>
<td>0.99</td>
</tr>
<tr>
<td>Serum estradiol (pmol/L)</td>
<td>62 (43-83)</td>
<td>56 (44-91)</td>
<td>0.94</td>
</tr>
<tr>
<td>Serum FSH (IU/L)</td>
<td>83 ± 32</td>
<td>82 ± 25</td>
<td>0.78</td>
</tr>
</tbody>
</table>

Values are given as mean ± SD, as median (25th – 75th percentile) or as number (n). intranasal, spray containing 175 μg 17β-estradiol (E2) and 275 μg norethisterone; oral, capsule containing 1 mg E2 and 0.5 mg norethisterone acetate

P-value for t-test (for amenorrhea and serum estradiol after log-transformation) or χ²-test for between-group differences.
significant (P<0.05). In week 52 the increase from baseline in both groups was non-significant (19.8% in the oral and 4.2% in the intranasal group).

Overall, we found significant (P<0.001) reductions in both groups in sVCAM, sICAM and sE-selectin (Table II, Figure 2b). These were larger in the oral compared with the intranasal group (P<0.01 for sVCAM and sICAM, and P<0.001 for sE-selectin). After 12 weeks of treatment the reductions in the intranasal and the oral group were -9.6% and -14.6% in sVCAM, -9.5% and -14.5% in sICAM, and -8.8% and -12.3% in sE-selectin, respectively (all P<0.001 versus baseline). Reductions persisted until week 52.

---

**Figure 1. Trial profile**

n, number of subjects.

intranasal, spray containing 175 mg E₂ and 275 mg norethisterone; oral, capsule containing 1 mg E₂ and 0.5 mg norethisterone acetate.
At baseline, there was a significant correlation between CRP and BMI (r=0.29, P<0.01) and between values of sE-selectin and sVCAM (r=0.30, P<0.01). There were no other significant correlations among the baseline values of CRP, sVCAM, sICAM and sE-selectin. Changes in sVCAM and sICAM, and in sVCAM and sE-selectin were positively correlated at week 12 (r=0.45, P<0.001 and r=0.36, P=0.001, respectively) and this persisted at week 24 and 52. Changes in CRP did not correlate with changes in adhesion molecules or with changes in SHBG.

Re-analysis after exclusion of those women who reported an infection in the weeks before blood sampling or with a CRP concentration ≥10 mg/L (intranasal n=4, oral n=6) revealed highly similar results (data not shown).

Smokers had significantly (P<0.05) higher baseline values of sICAM than non-smokers. Between-group differences in changes in CRP and adhesion molecules remained highly similar when smoking was added as an extra covariate in ANCOVA. Changes within the treatment groups did not differ between smokers and non-smokers (data not shown).

### Table II. Concentrations of inflammation parameters

<table>
<thead>
<tr>
<th></th>
<th>baseline</th>
<th>week 12</th>
<th>week 24</th>
<th>week 52</th>
<th>% Δ 0 - 52</th>
<th>P-value(^a)</th>
<th>P-value(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsCRP (mg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>intranasal</td>
<td>0.83 (0.8-1.98)</td>
<td>0.96 (0.41-1.66)</td>
<td>1.02 (0.42-2.46)</td>
<td>0.94 (0.36-1.82)</td>
<td>4.2 (-18.0 to 32.4)</td>
<td>0.20</td>
<td>0.001</td>
</tr>
<tr>
<td>oral</td>
<td>0.81 (0.42-1.18)</td>
<td>1.94 (0.63-3.45)</td>
<td>1.22 (0.55-3.43)</td>
<td>0.94 (0.47-2.47)</td>
<td>19.8 (-9.2 to 58.0)</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>P-value(^a)</td>
<td>0.44</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sVCAM (mg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>intranasal</td>
<td>756 ± 162</td>
<td>682 ± 155**</td>
<td>693 ± 162**</td>
<td>704 ± 168**</td>
<td>-6.3 (-9.5 to -3.2)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>oral</td>
<td>755 ± 120</td>
<td>643 ± 109**</td>
<td>645 ± 112**</td>
<td>657 ± 120**</td>
<td>-12.9 (-15.8 to -10.0)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>P-value(^a)</td>
<td>0.97</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sICAM (mg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>intranasal</td>
<td>509 ± 141</td>
<td>457 ± 125**</td>
<td>477 ± 129*</td>
<td>480 ± 146*</td>
<td>-7.1 (-11.4 to -2.6)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>oral</td>
<td>511 ± 109</td>
<td>436 ± 91**</td>
<td>430 ± 97***</td>
<td>437 ± 101**</td>
<td>-14.7 (-17.2 to -12.2)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>P-value(^a)</td>
<td>0.95</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sE-selectin (mg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>intranasal</td>
<td>54 (36-64)</td>
<td>46 (34-59)**</td>
<td>50 (36-63)</td>
<td>49 (36-62)*</td>
<td>-5.3 (-9.4 to -1.2)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>oral</td>
<td>57 (43-74)</td>
<td>44 (30-91)**</td>
<td>43 (32-57)**</td>
<td>45 (28-60)**</td>
<td>-20.7 (-25.5 to -16.0)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>P-value(^a)</td>
<td>0.36</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values at baseline and at week 12, 24 and 52 as mean ± SD or median (25th – 75th percentile), %Δ is percentage change from baseline in week 52 given as (geometric) mean (95% confidence interval).

Intranasal = spray containing 175 μg E2 and 275 μg norethisterone; oral = capsule containing 1 mg E2 and 0.5 mg norethisterone acetate; hsCRP = high sensitive C-reactive protein; s = soluble; VCAM = vascular cell adhesion molecule; ICAM = intercellular adhesion molecule.

\(^a\)P-value for unpaired t-test for comparison between the groups
\(^b\)Analysis of covariance for repeated measurements over 52 weeks with the baseline value of the parameter under consideration as constant covariate for between-group differences in change
\(^c\)Analysis of variance for repeated measurements for within-group changes over the 52 weeks

\*P<0.01 and \**P<0.001 for paired t-test versus baseline
In this study we investigated the effect of intranasal administration of combined estrogen plus progestogen therapy on various markers of inflammation, which are indicators of atherosclerosis. Intranasally administered E₂ is rapidly absorbed and induces very steep and short peaks in serum levels. This pulsed exposure affects the sensitivity of some target tissues to estrogen.²⁵ In endothelial cells a similar effect was observed after pulsed E₂ exposure to that found after continuous exposure. However it appeared to be induced through non-genomic pathways rather than the genomic pathways during continuous exposure.²⁶ An important potential advantage of the difference in

![Figure 2. Percentage changes from baseline](image-url)

Figure 2. Percentage changes from baseline
s, soluble; VCAM, vascular cell adhesion molecule; ICAM, intercellular adhesion molecule; intranasal, spray containing 175 μg E₂ and 275 μg norethisterone; oral, capsule containing 1 mg E₂ and 0.5 mg norethisterone acetate
Percentage change from baseline: *P<0.05, **P<0.01 and ***P<0.001
Between-group difference in percentage change from baseline: †P<0.05, ††P<0.01 and ‡P<0.001

Discussion

In this study we investigated the effect of intranasal administration of combined estrogen plus progestogen therapy on various markers of inflammation, which are indicators of atherosclerosis. Intranasally administered E₂ is rapidly absorbed and induces very steep and short peaks in serum levels. This pulsed exposure affects the sensitivity of some target tissues to estrogen. In endothelial cells a similar effect was observed after pulsed E₂ exposure to that found after continuous exposure. However it appeared to be induced through non-genomic pathways rather than the genomic pathways during continuous exposure. An important potential advantage of the difference in
mechanism might be low stimulation of the breast and endometrium. In the current study, as well as in a previous study, fewer women (P<0.01) reported breakthrough bleeding and mastalgia in the intranasal group. Furthermore, in the current study fewer women in the intranasal group, compared to the oral group, discontinued the study because of adverse events.

Improvement of climacteric complaints and 24-hour exposure to E₂ after intranasal administration of 300 μg E₂ was shown to be similar when compared to oral 2 mg E₂ and transdermal 50 μg E₂. Bioavailability of E₂ was more stable after intranasal than after oral administration. NETA is rapidly hydrolysed into the active hormone NET, and equivalent quantities of NETA and NET provide similar pharmacokinetic profiles for NET concentrations (unpublished data). Therefore, NET was used in the intranasal spray.

In the oral E₂/NETA group we found a significant 64% increase in CRP and significant decreases in sICAM, sVCAM and sE-selectin. During oral HT, most placebo-controlled studies report an increase in CRP and a decrease in adhesion molecules. An increase in CRP has been found to occur within 2 weeks after start of oral HT. The magnitude of the increase in CRP appears to be dose-related and even a decrease during ultra-low dose E₂ (0.25 mg) has been described. Addition of a progestogen can attenuate the increase in CRP depending on the type of progestogen used.

In the intranasal E₂/NET group, we are the first to report no significant increase in CRP and significant decreases in soluble adhesion molecules. Until now only one study has reported the effects of intranasal estrogen therapy on inflammation parameters, finding no increase in CRP after 6 months of unopposed intranasal E₂. As to other non-oral regimens, transdermal hormone therapy is the most widely studied. Compared with placebo, most studies reported changes in CRP during various transdermal E₂ regimens to be non-significant in healthy postmenopausal women. However, one study in women with type 2 diabetes reported a decrease in CRP during transdermal E₂ plus oral NETA. In general, adhesion molecule levels were reported to be decreased or unchanged.

The increase in CRP as well as the decrease in adhesion molecule levels was larger in the oral than in the intranasal group. This was also found in studies making a direct comparison between oral and transdermal regimens, including one study comparing oral and transdermal E₂/NETA.

Because cigarette smoking is an important cause of endothelial dysfunction, the effects of smoking were evaluated in this study. The difference in sICAM at baseline between smokers and non-smokers did not influence the results as smokers were equally divided among the oral and the intranasal group. The finding that changes in CRP and adhesion molecules were not affected by smoking status has been reported earlier.

It has yet to be determined what this increase in CRP levels during oral HT reflects. CRP appears to have a dualistic role in inflammation. On the one hand, it is a non-specific marker of inflammation, with hscRP reflecting the amount of infiltrate in atherosclerotic plaques. On the other hand, CRP appears to be able to aggravate an inflammatory response in the vascular wall by itself: exposure to CRP has been shown to induce expression of adhesion molecules on human endothelial cells and a higher rate of thrombotic occlusion was observed in the presence, rather than in the absence, of CRP in a mouse model. CRP appears to contribute to inflammation through complement activation, as was seen in ischaemic myocardium after infarction and in early atherosclerotic coronary
arteries. In both scenarios, the increase in CRP levels is an unwanted effect of oral HT, which can be avoided by administration by the non-oral route.

The absence of an increase in interleukin-6 (IL-6) and tumour necrosis factor α (TNFα), both important promoters of CRP synthesis in response to inflammation, provides evidence that counters the hypothesis that HT directly induces an inflammatory state. Furthermore, in vitro and animal studies have not demonstrated any pro-inflammatory response to HT, and the reduction in adhesion molecule levels during oral HT suggests an anti-inflammatory effect. Also, when similar increases in plasma E2 levels were obtained during oral and transdermal HT, CRP levels were raised during oral, but not so during transdermal treatment. An increase in plasma SHBG levels during oral HT is regarded as an indicator for estrogenic effects on hepatic protein synthesis. The absence of a correlation between the change in CRP and SHBG which we found has been described before and argues against the suggestion that the increase in CRP is just a hepatic first pass effect.

Hepatic production of a pro-inflammatory factor during oral HT plays a role in an interesting hypothesis to unify the apparently cardioprotective effect of HT in observational studies and an increased risk of CHD during the first year of HT in predominately late postmenopausal women in the Heart and Estrogen/progestin Replacement Study (HERS) and in the Women's Health Initiative trial (WHI). In late postmenopausal women, who most likely have more extensive vulnerable plaques, hepatic production of prothrombotic and pro-inflammatory factors may outweigh potentially beneficial effects on lipids and anticoagulant factors, therefore inducing plaque instability. However, in early postmenopausal women, in whom less vulnerable plaques are present, decreased plaque production after start of HT leads to a decrease in CHD. The temporary increase in CRP levels we found in the oral group during the first 24 weeks has been described previously. We can only speculate about the mechanisms of this later decrease in CRP-levels. Among the possibilities is the later onset of CRP-lowering mechanisms such as the following: (i) clearance, in association with the increased clearance of LDL with which CRP appears to interact closely; (ii) inhibition of protein production in general, as reflected in lower albumin levels; or (iii) a decrease in CRP-levels due to raised SHBG-levels. This temporary effect may account for the early CHD risk increase reported in the large randomised controlled trials.

One of the strengths of our study is the randomized, double-blind study design and the 1-year duration including the measurement of short-term effects after 3 months therapy. The study also has limitations. We did not include an untreated or placebo control group. However, we compared the effects of the new intranasal spray with a reference product, the effects of which were found to be highly similar to those observed in placebo-controlled studies.

In conclusion, in this study in healthy postmenopausal women, intranasal continuous combined E2/NET therapy did not significantly increase CRP levels, in contrast to the increase observed in the oral group within the first year of use. Both intranasal and oral therapy lowered adhesion molecules; however the effects were more pronounced in the oral group. These findings suggest a favourable effect of intranasal postmenopausal hormone therapy compared to oral therapy.

Acknowledgements
We thank all women who participated in the study; C Klipping, MD, Dinox Medical Investigations, Nijmegen, and MO Verhoeven, MD, and TE Vogelvang, MD, PhD, VU University Medical Center, Amsterdam, for logistical and technical assistance; A Kok, Department of Clinical Chemistry, VU
University Medical Center, Amsterdam, for laboratory assistance. This study was supported by a research grant from the Institut de Recherches Internationales Servier (I.R.I.S., Courbevoie, France) to the Biocare Foundation (grant no 01-049).

References


Less effect of intranasal than oral hormone therapy on factors associated with venous thrombosis risk in healthy postmenopausal women

Majoie Hemelaar
Jan Rosing
Peter Kenemans
M Christella LGD Thomassen
Didi DM Braat
Marius J van der Moeren

Arteriosclerosis Thrombosis and Vascular Biology 2006;26:1660-1666

The definitive version is available at:
http://atvb.ahajournals.org
http://atvb.ahajournals.org/cgi/content/full/26/7/1660
Abstract

Objective: To compare the effects of intranasal and oral administration of 17β-estradiol (E₂) and norethisterone(acetate) [NET(A)] in healthy postmenopausal women on activated protein C (APC) resistance and other hemostatic parameters associated with venous thrombosis.

Methods and results: In this 2-center, randomized, double-blind, 1-year trial, 90 postmenopausal women (56.6 ± 4.7 years of age) daily received either an intranasal spray with 175 μg/275 μg E₂/NET (n=47) or oral 1 mg/0.5 mg E₂/NETA (n=43). Normalized APC sensitivity ratios (nAPCsr) were determined with a thrombin generation-based APC resistance test. After 1 year, the increase in nAPCsr was smaller in the intranasal than in the oral group: 11% (95%CI, 1% to 22%) versus 53% (95%CI, 37% to 72%). Overall, the decrease in antithrombin and increase in prothrombin fragment 1+2 (F1+2) were smaller, and the decrease in free protein S larger in the intranasal compared with the oral group after 1 year. In both groups, the decreases in protein C and prothrombin, and the increase in D-dimer were similar.

Conclusion: Compared with oral E₂/NETA therapy, intranasal administration of E₂/NET had less effect on APC resistance and on a number of other parameters associated with venous thrombosis. This observation suggests the possibility of a lower venous thrombosis risk for intranasal E₂/NET compared with oral therapy.
Introduction

Use of oral postmenopausal hormone therapy (HT) is associated with a 2- to 4-fold increased risk of venous thromboembolism (VTE),\textsuperscript{1-3} with the highest risk during the first year of treatment. Although literature regarding possible risk differences between oral and transdermal is conflicting,\textsuperscript{1,3-5} the largest case-control study performed so far\textsuperscript{3} suggested that women on HT with transdermal estradiol (E\textsubscript{2}) may not be exposed to an increased risk of VTE (odds ratio [OR] 0.9; 95%CI, 0.5 to 1.6).

In 1993, Dahlbäck et al.\textsuperscript{6} discovered that activated protein C (APC) hardly prolonged the clotting of plasma in members of families with multiple thromboembolic events. This defect, called APC resistance, which was attributed to a mutation in the factor V (FV) gene\textsuperscript{7} (FV Leiden), appeared to be a major risk factor of venous thrombosis.\textsuperscript{8} Later, it was shown that APC resistance in the absence of FV Leiden is also associated with an increased VTE risk.\textsuperscript{9} APC resistance in individuals without FV Leiden is particularly observed during use of combined oral contraceptives (OCs),\textsuperscript{10,11} during pregnancy,\textsuperscript{11} and while using oral HT.\textsuperscript{11-15} Compared with oral HT, HT with transdermal E\textsubscript{2} has a much smaller effect on APC resistance.\textsuperscript{14,15}

Important other risk factors for the development of VTE are deficiencies in protein C, protein S and antithrombin, and elevated plasma levels of factor VIII (FVIII) and prothrombin\textsuperscript{16}. Also, elevated levels of prothrombin fragment 1+2 (F1+2) and D-dimer, both reflecting ongoing coagulation, have been observed in individuals with an increased VTE risk.\textsuperscript{17-21}

Oral HT induces changes in the plasma levels of coagulation factors that are indicative of the existence of a prothrombotic condition.\textsuperscript{22} Although it has been shown that HT with transdermal estradiol has less effect on hemostatic parameters than oral HT,\textsuperscript{14,23,24} limited data are available for other forms of non-oral hormone therapy.

An intranasal spray has shown to be effective in climacteric symptom relief and a well tolerated alternative route for E\textsubscript{2} administration (Aerodiol®, Servier, Courbevoie, France)\textsuperscript{25} with an attractive theoretical advantage of less stimulation of the breast and endometrium,\textsuperscript{26} leading to less breakthrough bleeding and mastalgia.\textsuperscript{25} In addition to the E\textsubscript{2}-only spray, an intranasal spray for continuous combined 17β-estradiol plus norethisterone (E\textsubscript{2}/NET) administration has been developed. Because the hepatic metabolism is largely bypassed, it is plausible that, like HT with transdermal estradiol,\textsuperscript{3} intranasal administration may have limited effect on VTE risk.

Because no data are available on the VTE risk during intranasal HT, we investigated the effect of the intranasal E\textsubscript{2}/NET spray on APC resistance and on the plasma levels of proteins that are associated with an increased risk of VTE. We compared the effects of the intranasal spray with those of low-dose continuous combined oral E\textsubscript{2}/NET acetate (NETA) therapy. This was the objective of a substudy among participants in 2 Dutch centers, in a large international, randomized, double-blind, double-dummy study, with endometrial safety as primary endpoint.

Materials and methods

Participants

Healthy postmenopausal women aged 40 to 75 years were recruited from gynecological outpatient clinics of 2 centers in the Netherlands and through advertisements in local newspapers.
Specific indications for HT use were not required, but women were willing to participate in the study because of some kind of climacteric complaints. All women were nonhysterectomized and had their last menstrual period at least two years before inclusion and had serum levels of E2 <30 pg/mL and of follicle stimulating hormone (FSH) >30 mIU/mL. All participants had a normal cervical smear and mammography within 12 months before inclusion. A normal transvaginal ultrasound and an endometrial biopsy without hyperplasia or polyps, were required, as well as blood tests (lipids, liver enzymes, kidney function, glucose and thyroid stimulating hormone) without any clinically relevant abnormalities. At screening, all participants had plasma levels of total cholesterol ≤8.0 mmol/L and triglycerides ≤3.0 mmol/L. Exclusion criteria were a body-mass index (BMI) >32 kg/m², any contra-indication for use of estrogen or progestogen, any ear-nose-throat disease that might interfere with intranasal drug administration, and concomitant use of any treatment for menopausal symptoms, chronic treatment liable to interfere with the coagulation profile, treatment liable to interfere with intranasal administration, enzyme inducers and systemic vasoconstrictors. The present study was done in a subset of women who were not taking lipid-lowering drugs, and who had either never used HT or had a wash-out of previous HT of at least 6 weeks before the baseline visit.

All participants gave written informed consent before participation in the trial, which was conducted in accordance with the ethical principles stated in the Declaration of Helsinki, with Good Clinical Practice, and was approved by central and local institutional review boards.

**Study design**

This study was performed in a subgroup of 90 women who were included in 2 centers in the Netherlands, as part of a large international, randomized, double-blind, double-dummy study with 2 parallel treatment arms including 954 women. Eligible women were randomized to daily either one intranasal spray containing a fixed dose of 175 μg E2 plus 275 μg NET (S21405, Servier, Coubevoie, France) and one placebo capsule (intranasal E2/NET) or one capsule containing 1 mg E2 plus 0.5 mg NETA (Activelle®, Novo Nordisk, Bagsvaerd, Denmark) and one placebo spray (oral E2/NETA). Study medication was manufactured, packaged and labeled by the Institut de Recherches Internationales Servier (Courbevoie, France). Placebo and active treatments were identical in appearance and smell. Centralized computerized subject randomization was done by an Interactive Voice Response System in blocks of 12 (6 active spray and 6 active capsules) per center. Treatment was administered for 52 weeks.

Throughout the whole study period, all participants, clinical investigators and laboratory personnel were blinded for the study medication. Unblinding was done after all data were collected in the database.

**Hemostatic parameters**

For assessment of hemostatic parameters, venous blood samples were taken at baseline and in week 12 and 52. After fasting and refraining from smoking for at least 10 hours and no alcohol intake for at least 24 hours, blood samples were taken between 8.00 and 10.00 a.m. After 20 minutes of rest, blood was collected into pre-cooled tubes (Becton Dickinson, Plymouth, United Kingdom) containing sodium citrate, theophylline, adenosine and dipyridamole (CTAD) for FVIII activity or 0.129 M sodium citrate (for protein C, free protein S, antithrombin, F1+2 and D-dimer) or into tubes at room-temperature containing 0.129 M sodium citrate (for nAPCsr, prothrombin and the test for the FV
Leiden mutation). After blood collection, pre-cooled tubes were immediately placed on ice. Within one hour after collection, plasma was separated by centrifugation at 2000 g for 30 minutes at 4°C (for blood collected into pre-cooled tubes) or at 20°C (for blood collected into tubes at room temperature). Plasma was divided into aliquots, snap-frozen and stored at -80°C until analysis. All samples of a given subject were assayed within a single run.

Normalized APC sensitivity ratios (nAPCsr) were determined with the endogenous thrombin potential (ETP)-based APC-resistance test, in which the effect of APC on the time integral of thrombin generation (ETP) is quantified. A normal plasma pool consisting of 45 men and 27 women (all without OCs/HT/pregnancy) with a mean age of 40 ± 8.9 years was used to normalize the APCsr. In this assay, women (without OCs/HT/pregnancy) generally have a nAPCsr between 1 and 2, whereas the mean nAPCsr of men is less than 1. Protein C activity was measured with the Coamatic protein C activity kit (Chromogenix, Mölndahl, Sweden), free protein S antigen with the Automated latex ligand immunoassay, IL Test Free Protein S (Instrumentation Laboratory, Lexington, MA, USA), antithrombin with the Coamatic antithrombin test (Chromogenix, Mölndahl, Sweden), FVIII activity with the Coatest Factor VIII (Chromogenix, Mölndahl, Sweden), prothrombin with the ecarin activation test, F1+2 with an ELISA (Enzygnost, Dade Behring, Marburg, Germany) and D-dimer with an ELISA (TintElize D-dimer, Biopool, Umea, Sweden). All women were tested for the FV Leiden mutation with a functional test which fully discriminates between homozygous, heterozygous and non-carriers of the mutation.

Serum levels of E2 and of FSH were measured with an electrochemiluminiscence immunoassay (ECLIA, Roche Diagnostics, Basel, Switzerland), serum sex-hormone binding globulin (SHBG) levels were measured using an immunoradiometric assay (IRMA, Orion Diagnostica, Espoo, Finland).

Statistical analyses

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) 11.5 for Windows (SPSS Inc., Chicago, IL, USA). Values of the hemostatic parameters are given as mean ± standard deviation or as median (25th–75th percentile) if skewed. Percentage changes from baseline are given as mean (95%CI) or as geometric mean (95%CI) if the changes had a skewed distribution and for D-dimer as median (25th–75th percentile) because changes remained skewed after log-transformation.

We used standard parametric tests. If variables were skewed, analyses were done after log-transformation. Within-group changes over time were tested using analysis of variance (ANOVA) for repeated measurements. For between-group comparisons we used analyses of covariance (ANCOVA) for repeated measurements, with the baseline value of the variable under consideration as covariate. Non-parametric tests were used for comparison between groups of smoking at baseline (χ²-test) and percentage changes in D-dimer (Mann-Whitney U-test).

Only data from women, of whom data were available at baseline and at least at one other time point, were used for analyses; for AN(C)OVA for repeated measurements the last-observation-carried-forward procedure for the missing values was applied.

Sample size calculation for this cardiovascular substudy was based on changes in nAPCsr. To find a 35% difference in change in nAPCsr between the groups with a standard deviation in percentage change of 75%, using a power of 80% and an α of 5% (2-sided), 37 evaluable women would be required in each group.
Results

Participants

Between September 2001 and June 2002, 125 women were screened in the 2 participating centers, of whom 94 women were randomized. Because 4 women either had no washout from their previous HT (n=3) or used lipid-lowering drugs (n=1), 90 women were eligible for the current substudy. The last patients completed the study in May 2003. No differences in baseline demographic characteristics were found between the two groups (Table I).

Two women in the intranasal group discontinued the study versus ten women in the oral group (P<0.01) (Figure 1). Premature study discontinuation was mainly related to the occurrence of an adverse event. No women stopped because of coronary or cerebrovascular events. In the intranasal group, one woman discontinued in week 11 because of the occurrence of deep venous thrombosis which, in retrospect, most likely was already present before study entry, and another woman discontinued because of vaginal candidiasis. One woman in the intranasal group was excluded from analyses after week 12 as she started preventive anticoagulant therapy because of a family history of cerebrovascular disease. In the oral group, one woman discontinued in week 36 because of clinical symptoms of venous thrombosis which, however, could not be confirmed ultrasonographically. This woman was one of the four women who carried the FV Leiden mutation. Furthermore, one woman in the oral group stopped because of the detection of breast cancer, which, in retrospect, was already present at the mammography before study entry. Other reasons for discontinuation in the oral group

<table>
<thead>
<tr>
<th>Table I. Baseline characteristics</th>
<th>Intranasal</th>
<th>Oral</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>47</td>
<td>43</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>57.5 ± 5.2</td>
<td>55.9 ± 3.9</td>
</tr>
<tr>
<td>Amenorrhoea (months)</td>
<td>74 (51-123)</td>
<td>73 (45-106)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>68.4 ± 9.2</td>
<td>68.5 ± 9.3</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>24.8 ± 3.3</td>
<td>25.2 ± 3.6</td>
</tr>
<tr>
<td>Blood pressure (mmHg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>systolic</td>
<td>126 ± 17</td>
<td>121 ± 17</td>
</tr>
<tr>
<td>diastolic</td>
<td>80 ± 10</td>
<td>76 ± 11</td>
</tr>
<tr>
<td>Smoking n (%)</td>
<td>12 (27.7%)</td>
<td>11 (25.6%)</td>
</tr>
<tr>
<td>Previous HT use n (%)</td>
<td>14 (32.6%)</td>
<td>12 (27.7%)</td>
</tr>
<tr>
<td>Washout from HT (weeks)</td>
<td>16.0 (12.9-21.6)</td>
<td>15.7 (13.7-20.7)</td>
</tr>
<tr>
<td>Serum cholesterol (mmol/L)</td>
<td>6.1 ± 1.1</td>
<td>6.1 ± 0.8</td>
</tr>
<tr>
<td>Serum triglycerides (mmol/L)</td>
<td>1.15 (0.92-1.54)</td>
<td>0.99 (0.76-1.48)</td>
</tr>
<tr>
<td>Serum estradiol (pmol/L)</td>
<td>62 (43-83)</td>
<td>56 (44-91)</td>
</tr>
<tr>
<td>Serum FSH (IU/L)</td>
<td>83 ± 32</td>
<td>82 ± 25</td>
</tr>
</tbody>
</table>

Values are given as mean ± SD, as median (25th–75th percentile) or as number (n). Previous HT use, use of hormone therapy in year before inclusion in the study; FSH, follicle-stimulating hormone; intranasal, spray containing 175 μg E₂ and 275 μg NET; oral, capsule containing 1 mg E₂ and 0.5 mg NETA.
were vaginal bleeding, fatigue, lack of efficacy, withdrawal of consent, nasal complaints, arthralgia, abdominal complaints and headache.

Analyses were based on 86 women (46 in the intranasal and 40 in the oral group) of whom values at baseline and at least at one other time point were available.

Hemostatic parameters

No women in the intranasal group compared with four women in the oral group were heterozygous carriers of the FV Leiden mutation. Baseline levels of protein C were lower in the intranasal than in the oral group. At baseline, there were no other significant differences between the two groups (Table II). FV Leiden carriers had higher baseline nAPCsr (range 3.03 to 6.97) which remained higher throughout the study period.

The increase in nAPCsr in the intranasal group was smaller (P<0.001) than in the oral group. After 52 weeks of treatment, the mean increase of the nAPCsr was 11.2% (95% confidence interval [95%CI] 1.0% to 22.3%) in the intranasal group and 53.8% (95%CI 37.1% to 72.5%) in the oral group. Increases in nAPCsr were already apparent after 12 weeks of treatment: 15.5% (95%CI 8.3% to 23.2%) in the intranasal and 51.3% in the oral group (95%CI 39.0% to 64.7%) (Figure 2).
<table>
<thead>
<tr>
<th>Hemostatic Parameter</th>
<th>Baseline</th>
<th>Week 12</th>
<th>Week 52</th>
<th>% Δ 0–52</th>
<th>P-value for between-group test†</th>
<th>P-value for within-group test‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>nAPCsr</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intranasal</td>
<td>1.60 (1.24-2.07)</td>
<td>1.87 (1.34-2.25)</td>
<td>1.89 (1.38-2.15)</td>
<td>11.2 (1.0 to 22.3)</td>
<td>&lt;0.001</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Oral</td>
<td>1.79 (1.20-2.14)</td>
<td>2.40 (2.05-3.03)</td>
<td>2.71 (2.06-3.10)</td>
<td>53.8 (37.1 to 72.5)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Difference*</td>
<td>0.27</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein C (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>Intranasal</td>
<td>117 ± 18</td>
<td>103 ± 15</td>
<td>103 ± 17</td>
<td>-11.1 (-13.5 to -8.7)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Oral</td>
<td>127 ± 25</td>
<td>111 ± 23</td>
<td>110 ± 22</td>
<td>-11.6 (-13.9 to -9.3)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Difference*</td>
<td>0.04</td>
<td>0.09</td>
<td>0.77</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Free protein S (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Intranasal</td>
<td>90 ± 12</td>
<td>87 ± 12</td>
<td>87 ± 11</td>
<td>-2.2 (-4.3 to -0.04)</td>
<td>&lt;0.001</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Oral</td>
<td>92 ± 12</td>
<td>92 ± 11</td>
<td>94 ± 13</td>
<td>2.2 (-0.9 to 5.2)</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td>Difference*</td>
<td>0.59</td>
<td>0.02</td>
<td>0.02</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antithrombin (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Intranasal</td>
<td>110 ± 13</td>
<td>104 ± 12</td>
<td>104 ± 12</td>
<td>-5.7 (-7.8 to -3.6)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Oral</td>
<td>115 ± 11</td>
<td>103 ± 10</td>
<td>104 ± 10</td>
<td>-10.2 (-12.0 to -8.3)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Difference*</td>
<td>0.06</td>
<td>0.92</td>
<td>0.02</td>
<td></td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>FVIII (IU/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.95</td>
<td>0.09</td>
</tr>
<tr>
<td>Intranasal</td>
<td>173 ± 49</td>
<td>165 ± 48</td>
<td>165 ± 47</td>
<td>-1.9 (-6.6 to 2.8)</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>Oral</td>
<td>172 ± 45</td>
<td>168 ± 43</td>
<td>157 ± 43</td>
<td>-5.0 (-10.4 to 0.5)</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>Difference*</td>
<td>0.96</td>
<td>0.42</td>
<td>0.38</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prothrombin (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.70</td>
<td></td>
</tr>
<tr>
<td>Intranasal</td>
<td>101 ± 12</td>
<td>95 ± 10</td>
<td>95 ± 10</td>
<td>-5.2 (-7.0 to -3.4)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Oral</td>
<td>100 ± 13</td>
<td>95 ± 11</td>
<td>96 ± 12</td>
<td>-4.8 (-7.1 to -2.4)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Difference*</td>
<td>0.73</td>
<td>0.79</td>
<td>0.78</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1+2 (nmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.05</td>
<td>0.99</td>
</tr>
<tr>
<td>Intranasal</td>
<td>0.9 (0.8-1.2)</td>
<td>1.0 (0.8-1.4)</td>
<td>1.0 (0.8-1.3)</td>
<td>5.8 (-4.2 to 15.8)</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>Oral</td>
<td>0.9 (0.7-1.1)</td>
<td>1.0 (0.9-1.3)</td>
<td>1.1 (0.9-1.3)</td>
<td>19.0 (5.8 to 32.2)</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Difference*</td>
<td>0.29</td>
<td>0.52</td>
<td>0.11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-dimer (μg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.41</td>
<td></td>
</tr>
<tr>
<td>Intranasal</td>
<td>41 (23-83)</td>
<td>62 (37-106)</td>
<td>57 (29-89)</td>
<td>16.1 (-21.0 to 112.9)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Oral</td>
<td>50 (23-81)</td>
<td>82 (44-148)</td>
<td>53 (23-118)</td>
<td>17.6 (-28.5 to 77.2)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Difference*</td>
<td>0.82</td>
<td>0.82</td>
<td>0.71</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The decrease in protein C was similar (difference +0.5% [95%CI -2.8% to 3.8%]) in both groups: 11.1% in the intranasal and -11.6% in the oral group in week 52. Free protein S was more decreased (difference -4.4% [95%CI -7.8 to -0.8]) in the intranasal group: -2.2% in week 52 than in the oral group (+2.2%). For antithrombin the decrease was less pronounced (difference +4.5% [95%CI 2.6% to 7.3%]) in the intranasal (-5.7%) than in the oral (-10.2%) group (Figure 3).

No significant difference in change was seen between the groups in FVIII (difference +3.1% [95%CI -3.9% to 10.2%]), although in week 12 a transient decrease (-3.7%) was observed in the intranasal group. In week 52, prothrombin was equally decreased (difference -0.4% [95%CI -3.3 to 2.5%]) in both groups: -5.2% in the intranasal and -4.8% in the oral group.

The increase in F1+2 in the oral group (19.0% in week 52) was larger (difference +13.2% [95%CI -2.8% to 29.1%]) than the non-significant increase in the intranasal group (5.8%). After 52 weeks the increase in D-dimer did not significantly differ between the groups, although in week 12 the increase was less pronounced in the intranasal group (29.8%) than in the oral group (50.7%) (Figure 3).
Results did not change when analyses were performed after exclusion of the four carriers of the FV Leiden mutation or when the presence of FV Leiden mutation was added as an additional covariate in the ANCOVA (data not shown). Also, exclusion of outliers in the other parameters did not affect the results.

Discussion

At present there are no reports on the risk of venous thrombosis associated with intranasal HT. The current study is the first to describe the effects of intranasal administration of combined estrogen plus progestogen therapy on hemostatic parameters that are associated with an increased risk of venous thrombosis.

Whereas use of oral postmenopausal HT is known to increase VTE risk,1,2,29 less is known about the risk of non-oral administration. So far, only four case-control studies,1,3-5 have investigated the association between VTE and HT with non-oral, i.e. transdermal E2. Scarabin et al,3 the largest of these studies, investigating healthy women who had their first idiopathic venous thrombosis, found no increased risk (OR [95%CI], 0.9 [0.5 to 1.6]) among users of HT with transdermal E2 compared with the increased risk (OR, 3.5 [1.8 to 6.8]) among users of oral HT. The other three studies included very small numbers of cases (two, five and seven) and two of them1,4 found a non-significant increase in VTE during HT with transdermal E2, however, smaller than for oral HT.1

Intranasally administered estradiol is rapidly absorbed and induces very steep and short peaks in serum E2 levels. Because of the lack of a first-pass liver effect, intranasally administered hormones

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Figure 3. Percentage change from baseline of the plasma levels of hemostatic parameters in week 12 and 52.

*significant difference between the groups; intranasal, spray containing E2/NET; oral, capsule containing E2/NETA; F1+2, prothrombin fragment 1+2.
may have less effect on plasma levels of coagulation factors than orally administered hormones. Hence, like has been suggested for HT with transdermal E_2, HT with intranasal E_2 may be associated with a lower risk of venous thrombosis. The dosages we compared have shown similar exposure to E_2 and NET, the active hormone of NETA (unpublished data, 2001).

Until now, the effect of intranasal E_2 on hemostatic parameters has been reported in only one study. Given with oral dydrogesterone in a sequentially combined fashion, 24 weeks of intranasal 300 μg E_2 did not show significant changes from baseline in antithrombin or D-dimer.

Increased resistance to APC is associated with an increased VTE risk. APC resistance was measured with the ETP-based APC-resistance test. This is a functional assay that, in contrast to the commonly used activated partial thromboplastin time (APTT) -based APC-resistance test, is very sensitive for changes in the levels of sex steroid hormones.

In this 1 year, randomized, double-blind study the increase in nAPCsr during intranasal E_2/NET therapy (11%) was significantly less than the increase observed during oral E_2/NETA therapy (54%). With respect to oral E_2/NETA therapy, our observations are in agreement with placebo-controlled studies with various oral HT regimens including E_2/NETA therapy. Literature on the effect of non-oral HT on APC resistance is only available for transdermal therapy. Compared with placebo and oral HT, HT with transdermal E_2 caused a smaller increase or had no significant effect on the nAPCsr.

Our study further shows that, among the antithrombotic proteins, protein C was similarly decreased in both groups, and free protein S showed a larger decrease and antithrombin a smaller decrease in the intranasal E_2/NET compared to the oral E_2/NETA group. For the comparison between oral HT and HT with transdermal E_2, previous studies generally reported no significant difference in changes in antithrombotic proteins. During oral HT plasma levels of anticoagulant proteins are significantly reduced, although in some studies, a transient decrease or no change of protein S was observed. During HT with transdermal E_2 both decreases and no changes of anticoagulant proteins were reported.

In the current study, intranasal E_2/NET and oral E_2/NETA decreased the plasma level of prothrombin, increased D-dimer to a similar extent, and had no effect on the FVIII level. A significant increase of F1+2 was only observed during oral E_2/NETA therapy. Our findings with oral E_2/NETA are largely in line with previous randomized studies in which HT using oral and transdermal E_2 were compared and with placebo-controlled studies in which the effect of oral E_2/NETA was investigated. During transdermal E_2, either unopposed, or combined with transdermal or oral progestogen, slight increases or no effect on FVIII, prothrombin, F1+2 or D-dimer were found.

The significantly smaller increase in nAPCsr in the intranasal group might reflect a lower risk for the development of VTE during intranasal HT. This would be in line with the observations that HT using transdermal E_2 induces a relatively small increase in the nAPCsr and may not increase the risk of VTE. Also in OC users there appears to be a good correlation between the nAPCsr and the risk of venous thrombosis. Women who use second-generation oral contraceptives are exposed to a lower thrombotic risk than third-generation pill users and their plasma is less APC resistant than that of third-generation pill users.

One of the strengths of our study is the randomized, double-blind study design and the 1 year duration, including the measurement of short-term effects after 3 months of therapy. The study also has limitations. We did not include an untreated or placebo control group. However, we compared the effects of the new intranasal spray with a well studied reference product, the effects of which
were found to be comparable with those observed in placebo-controlled studies. The study was not designed to detect differences in venous thrombotic risk or a possible interaction between FV Leiden and HT use.

In conclusion, in this study in healthy postmenopausal women, intranasal continuous combined E₂/NET therapy showed smaller changes in nAPCsr, antithrombin and F1+2 than oral E₂/NETA therapy. This might be indicative for a lower VTE risk during intranasal E₂/NET therapy when compared to oral therapy.

Acknowledgements

We thank all women who participated in the study. C Klipping, MD, Dinox Medical Investigations, Nijmegen, and MO Verhoeven, MD, and TE Vogelvang, MD, PhD, VU University Medical Center, Amsterdam, are acknowledged for logistical and technical assistance, and we thank EJP Magdeleyns, Department of Biochemistry, Maastricht University, Maastricht, and A Kok, Department of Biochemistry, VU University Medical Center, Amsterdam, for laboratory management.

This study was supported by a research grant from the Institut de Recherches Internationales Servier to the Biocare foundation (grant no 01-049).

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Haemostatic markers in healthy postmenopausal women during intranasal and oral hormone therapy: a randomized trial

Majoie Hemelaar
Peter Kenemans
C Erik Hack
Christine Klipping
Marius J van der Mooren

Menopause, in press

The definitive version is available at:
http://www.atherosclerosis-journal.com
http://www.atherosclerosis-journal.com/article/PII:S0021915006005880
Abstract

Objective: To study changes in the haemostatic balance during intranasal compared with oral administration of 17β-estradiol (E₂) and norethisterone (acetate) [NET(A)] in postmenopausal women. A wide range of markers of coagulation and fibrinolysis associated with coronary artery disease was tested.

Design: In a two-centre, randomized, double-blind, comparative trial, 90 healthy postmenopausal women (age 56.6 ± 4.7 yr) received daily continuous combined hormone therapy, either intranasally E₂/NET 175 μg/275 μg as a spray (n=47), or orally E₂/NETA 1 mg/0.5 mg as a capsule (n=43) for 1 year. Haemostatic markers were measured in blood samples taken at baseline and after 12, 24 and 52 weeks of treatment.

Results: After 52 weeks of treatment, changes in the intranasal group in markers of coagulation, (fibrinogen (-1.3%), FVII activity (-14.0%) and in F1+2 (+5.8%)), were significantly less (P<0.05) than the changes in the oral group in these parameters (-6.5%, -20.3% and +19.0%, respectively). Changes in FVIIa did not differ between the groups. Neither group showed significant changes in TAT. In the intranasal group, decreases in markers of fibrinolysis, (tPA (-10.4%) and PAI-1 Ag (-13.8%)), were significantly less (P<0.05) than the decreases in the oral group (-17.8% and -38.0%, respectively). Decrease in PAI-1 act and increases in D-dimer and PAP did not differ between the groups. No differences were found between the groups in homocysteine, which was overall unaltered in both groups.

Conclusions: During intranasal E₂/NET therapy changes in the coagulatory and fibrinolytic markers were to some extent less than those observed during oral therapy.
Introduction

The effect of postmenopausal hormone therapy (HT) on coronary heart disease (CHD) risk is a subject of debate. Evidence from observational studies suggested a cardioprotective effect of HT. However, randomized controlled trials did not confirm a reduction in cardiovascular risk by HT, neither among women with confirmed CHD nor in apparent healthy postmenopausal women without a history of CHD using oral conjugated equine estrogens alone or combined with medroxyprogesterone acetate. The effects of alternative regimens and administration routes have yet to be established. One secondary prevention trial found no protection during transdermal HT use. Until clinical endpoint studies in healthy postmenopausal women using alternative regimens are available, cardiovascular risk marker studies could be useful to understand mechanisms leading to clinical cardiovascular effects.

Plaque rupture and thrombus formation are known to play a major role in the development of ischemic heart disease. In addition to lipids and inflammation, the haemostatic balance contributes to this process. Homocysteine has a direct effect through interaction with haemostasis and the endothelium. Impaired fibrinolytic function, increased coagulability and hyperhomocysteinaemia are associated with increased cardiovascular risk in persons with cardiovascular disease as well as in healthy subjects. As for other cardiovascular markers, haemostatic parameters have demonstrated different effects of HT depending on dose, regimen and administration route.

In addition to the oral and the transdermal routes of administration, a spray is available for intranasal administration of estradiol (E₂) (Aerodiol®, Servier, Courbevoie, France). Intranasal E₂ has shown to be equally successful as tablets in relieving climacteric symptoms, but with fewer side effects. The aim of the present study was to investigate whether changes in markers of coagulation and fibrinolysis during use of an intranasal spray for continuous combined 17β-estradiol and norethisterone (E₂/NET) administration were less than those during oral continuous combined E₂ and norethisterone acetate (E₂/NETA). Nested within a large international, randomized, double-blind, double-dummy study, haemostatic parameters were studied as a secondary objective among participants in two Dutch centers.

Methods

Participants and study design

The study design has been described previously. In short, healthy non-hysterectomized postmenopausal women aged 40 to 75 years were recruited. All women had their last menstrual period at least two years before inclusion and had serum concentrations of estradiol (E₂) <110 pmol/L and of follicle stimulating hormone (FSH) >30 mIU/mL. At screening all participants had plasma levels of total cholesterol ≤8.0 mmol/L and triglycerides ≤3.0 mmol/L. Among the criteria for exclusion were: a body-mass index (BMI) >32 kg/m², any contra-indication for use of estrogen and/or progestogen, any ear-nose-throat disease that might interfere with intranasal drug administration, and concomitant use of any cardiovascular medication or treatment liable to interfere with the study medication. Women in this sub study had either no history of HT use, or had a wash-out period after previous HT use of at least 6 weeks before the baseline visit.
All participants gave written informed consent before inclusion in the trial, which was conducted in accordance with the ethical principles stated in the Declaration of Helsinki, with Good Clinical Practice, and with the approval of the central and local institutional review boards.

This study was performed in 90 women, who were included in two centers in the Netherlands, as part of a large international, randomized, double-blind, double-dummy study with 2 parallel treatment arms including 954 women in total. Eligible women were randomized to daily either one intranasal spray containing a fixed dose of 175 μg E₂ and 275 μg NET (S21405, Servier, Courbevoie, France) and one placebo capsule (intranasal E₂/NET group), or one capsule containing 1 mg E₂ and 0.5 mg NETA (Activelle®, Novo Nordisk, Bagsvaerd, Denmark) and one placebo intranasal spray (oral E₂/NETA group). Placebos and active treatments were identical in appearance and smell. Centralized computerized subject randomization was done by an Interactive Voice Response System (I.V.R.S.) in blocks of 12 (6 active spray and 6 active capsules) per centre. Treatment was administered for 52 weeks.

Throughout the whole study period all participants, clinical investigators and laboratory personnel were blinded for the study medication. Unblinding was done after all data were collected in the database.

**Haemostatic parameters**

For assessment of concentrations of haemostatic parameters, venous blood samples were taken at baseline and in week 12, 24 and 52. After a period of fasting and non-smoking of at least 10 hours and no alcohol intake for at least 24 hours, blood samples were taken between 8.00 a.m. and 10.00 a.m. After 20 minutes of rest, blood was collected into tubes (Becton Dickinson, Plymouth, United Kingdom) at room temperature containing 0.129 M sodium citrate (for activated FVII (FVIIa), FVII activity (VIIact), thrombin-antithrombin (TAT) and plasmin-α2-antiplasmin (PAP) complexes), or into pre-cooled tubes containing 0.129 M sodium citrate (for fibrinogen, prothrombin fragment 1+2 (F1+2) and D-dimer), CTAD (for tissue-type plasminogen activator (tPA) antigen, plasminogen activator inhibitor-1 (PAI-1) antigen and PAI-1 activity) or K₃-EDTA (for homocysteine). After blood collection, pre-cooled tubes were immediately placed in ice. Within one hour after collection, plasma was separated by centrifugation at 2000 g for 30 minutes at 4°C (for pre-cooled tubes) or at 20°C (for tubes at room temperature). Plasma was divided into aliquots, snap-frozen and stored at -80°C until analysis. For each marker, all samples of a given subject were assayed within a single run.

Plasma levels of markers of coagulation were measured with the following techniques: fibrinogen according to Clauss (STA fibrinogen, Diagnostica Stago, Roche, Mannheim, Germany); activated factor VII (FVIIa) with a clotting assay of FVII (Staclot Vila-rTF, Diagnostica Stago, Roche, Mannheim, Germany); factor VII activity (FVIIact) with a clotting method (CryoCheck FVII Deficient Plasma, Precision Biologicals, Kordia, Leiden, The Netherlands); F1+2 fragment with ELISA (Enzygnost F1+2 micro, Dade Behring, Marburg, Germany); and TAT complexes with ELISA (Enzygnost TAT micro, Dade Behring, Marburg, Germany).

Plasma levels of markers of fibrinolysis were measured as follows: tPA with ELISA (Zymuthest, Hyphen Biomed, Andrésy, France); PAI-1 ag with ELISA (Elitest PAI-1, Hyphen Biomed); PAI-1 activity (PAI-1 act) with a chromogenic assay (spectrolyse / pl. PAI, Biopool International, Umea, Sweden); D-dimer with ELISA (TintElize D-dimer, Biopool International, Umea, Sweden); and PAP complexes with ELISA (TC PAP complex, Technoclone, Surrey, UK). Assays were performed according to manufacturer's instructions.
Homocysteine plasma levels were measured by fluorescence polarization immunoassay (Abbott IMx, Abbott Laboratories, Abbott Park, IL, USA).

The inter-assay coefficients of variation were: 2.0-3.8% for fibrinogen, 9% for FVIIa, 7.4% for FVIIact, 12.2% for F1+2, 6-9% for TAT, 7.1% for tPA, 3.0-8.1% for PAI-1 ag, 10% for PAI-1 act, 3-4% for D-dimer, <10% for PAP, and <4% for homocysteine.

Plasma levels of estradiol and of FSH were measured on the Roche E-170 Modular (Roche, Basel, Switzerland) using electrochemiluminiscence immunoassay (ECLIA).

Statistical analyses

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) 11.5 for Windows (SPSS Inc., Chicago, IL, USA). Concentrations of the haemostatic parameters are given as mean ± standard deviation or as median (25th – 75th percentile) if skewed. Percentage changes from baseline are given as mean (95% confidence interval [95%CI]), as geometric mean (95%CI) if the changes had a skewed distribution, or as median (25th – 75th percentile) if distribution remained skewed after “log”-transformation.

Statistical analyses were performed using standard parametric tests; if the variables were skewed, analyses were done after “log”-transformation. Non-parametric tests were used for comparison between groups of smoking at baseline ($\chi^2$-test) and of baseline values of homocysteine and percentage changes in D-dimer and PAP (Mann-Whitney U-test). Within-group changes over time were tested using analysis of variance (ANOVA) for repeated measurements and paired t-tests versus baseline. For between-group comparisons we used analyses of covariance (ANCOVA) for repeated measurements, with the baseline value of the variable under consideration as constant covariate, and unpaired t-test.

Only data from women, for whom data were available at baseline and at least at one other time point, were used for the analyses; for ANOVA and ANCOVA for repeated measurements the last-observation-carried-forward procedure for the missing values was applied.

Correlations were analyzed by calculation of the Pearson’s correlation coefficient.

Sample size calculation for this cardiovascular sub study was based on changes in normalized APC sensitivity ratio (nAPCsr). To find a 35% difference in change in nAPCsr between the groups with a standard deviation in percentage change of 75%, using a power of 80% and an $\alpha$ of 5% (two-sided), 37 evaluable women would be required in each group.

Results

Participants

Between September 2001 and June 2002, 125 women were screened in the two participating centers, of whom 94 women were randomized. As four women either had no wash-out from their previous HT (n=3) or used lipid-lowering drugs (n=1), 90 women were eligible for the current sub study. The last patients completed the study in May 2003. No differences in baseline demographic characteristics were found between the two groups (Table I).

Two women in the intranasal group discontinued the study versus ten women in the oral group ($P<0.01$) (Figure 1). Premature study discontinuation was mainly related to the occurrence of an adverse event. No women stopped because of coronary or cerebrovascular events.$^{13}$ Two women
discontinued because of (suspected) venous thrombosis. In one woman in the intranasal group, in week 11 a deep venous thrombosis was detected which, based on her history, likely was already present before study entry. One woman in the oral group developed clinical symptoms of venous thrombosis in week 36 which, however, could not be confirmed ultrasonographically. The latter women tested positive for the factor V Leiden mutation, the woman in the intranasal group did not have any additional risk factors. Furthermore, one woman in the oral group stopped because of the detection of breast cancer and one woman in the intranasal group was excluded from analyses after week 12 as she started preventive anticoagulant therapy because of a family history of cerebrovascular disease.

Analyses were based on 86 women (46 in the intranasal and 40 in the oral group) from who values at baseline and at least at one other time point were available.

### Haemostatic parameters

At baseline, plasma levels of FVIIa and FVII act were lower (P<0.05) in the intranasal than in the oral group, whereas other parameters did not show a difference (Table II).

Markers of coagulation: Overall, in the intranasal group, changes in fibrinogen (-1.3%), FVII activity (-14.0%) and in F1+2 (+5.8%) after 52 weeks, were significantly less (P<0.05) than the changes in the oral group in these parameters (-6.5%, -20.3% and +19.0%, respectively). Changes in FVIIa did not differ between the groups. Neither group showed significant changes from baseline in TAT (Figure 2a).

Markers of fibrinolysis: Overall, decreases in the intranasal group in tPA (-10.4%) and in PAI-1 Ag (-13.8%) after 52 weeks were significantly less (P<0.05) than the decreases in the oral group (-17.8% and -38.0%, respectively). Overall, decrease in PAI-1 act and increases in D-dimer and PAP did not differ between the groups. Percentage changes in week 12 in D-dimer and in PAP were smaller (P<0.05) in the intranasal (+29.8% and -6.6%, respectively) than in the oral group (+50.7% and +33.3%, respectively) (Figure 2b).

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### Table I. Baseline characteristics

<table>
<thead>
<tr>
<th></th>
<th>Intranasal</th>
<th>Oral</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>47</td>
<td>43</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>57.5 ± 5.2</td>
<td>55.9 ± 3.9</td>
</tr>
<tr>
<td>Amenorrhea (months)</td>
<td>74 (51–123)</td>
<td>73 (45–106)</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>24.8 ± 3.3</td>
<td>25.2 ± 3.6</td>
</tr>
<tr>
<td>Blood pressure</td>
<td></td>
<td></td>
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<tr>
<td>systolic (mmHg)</td>
<td>126 ± 17</td>
<td>121 ± 17</td>
</tr>
<tr>
<td>diastolic (mmHg)</td>
<td>80 ± 10</td>
<td>76 ± 11</td>
</tr>
<tr>
<td>Smoking n (%)</td>
<td>12 (27.7%)</td>
<td>11 (25.6%)</td>
</tr>
<tr>
<td>Serum cholesterol (mmol/L)</td>
<td>6.1 ± 1.1</td>
<td>6.1 ± 0.8</td>
</tr>
<tr>
<td>Serum estradiol (pmol/L)</td>
<td>62 (43–83)</td>
<td>56 (44–91)</td>
</tr>
<tr>
<td>Serum FSH (IU/L)</td>
<td>83 ± 32</td>
<td>82 ± 25</td>
</tr>
</tbody>
</table>

Values are given as mean ± SD, as median (25th–75th percentile) or as number (n).

FSH, follicle-stimulating hormone; intranasal, spray containing 175 μg 17β-estradiol (E2) and 275 μg norethisterone; oral, capsule containing 1 mg E2 and 0.5 mg norethisterone acetate
Homocysteine: No differences in changes were found between the groups. In both groups, overall changes from baseline in homocysteine did not reach statistical significance. However, homocysteine was transiently decreased in week 12 in the oral group (P<0.05) (Figure 2c).

Analyses of only women completing the study (n=78) revealed highly similar differences between the groups, except for the comparison for fibrinogen: instead of 0.04, the P-value was now exactly 0.05. Analyses without five women having a wash-out from previous HT of less than twelve weeks did not alter the results.

Correlations

The following correlations for changes at week 12 were found, which persisted throughout the study. Among the coagulation parameters: fibrinogen and F1+2 (r=-0.38, P<0.001) and FVIIa and FVII act (r=0.28, P<0.01). Among the fibrinolytic parameters: tPA and PAI-1 ag (r=0.54), tPA and PAI-1 act (r=0.42), PAI-1 ag and PAI-1 act (r=0.62), and D-dimer and PAP (r=0.41) (all correlations P<0.001). Between coagulation and fibrinolytic parameters: fibrinogen and tPA (r=0.36) and fibrinogen and PAP (r=0.40) (both P<0.001). Changes in homocysteine levels did not correlate with changes in any of the haemostatic parameters studied.
### Table II. Plasma levels of haemostatic markers

<table>
<thead>
<tr>
<th>Markers of coagulation</th>
<th>Baseline</th>
<th>Week 12</th>
<th>Week 24</th>
<th>Week 52</th>
<th>% Δ 0–52</th>
<th>ANCOVA †</th>
<th>ANOVA ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fibrinogen (g/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Intranasal</td>
<td>3.4 ± 0.5</td>
<td>3.4 ± 0.4</td>
<td>3.4 ± 0.6</td>
<td>3.4 ± 0.6</td>
<td>-1.3 (-4.6 to 2.2)</td>
<td>0.74</td>
<td></td>
</tr>
<tr>
<td>Oral</td>
<td>3.4 ± 0.5</td>
<td>3.6 ± 0.6</td>
<td>3.2 ± 0.6†</td>
<td>3.2 ± 0.6‡</td>
<td>-6.5 (-9.9 to -3.0)</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>P-value§</td>
<td>0.65</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.05</td>
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</tr>
<tr>
<td><strong>FVIIa (U/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.59</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intranasal</td>
<td>58 ± 22</td>
<td>52 ± 22†</td>
<td>50 ± 19¶</td>
<td>57 ± 28</td>
<td>-6.3 (-16.4 to 5.1)</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>Oral</td>
<td>71 ± 33</td>
<td>63 ± 27</td>
<td>55 ± 22¶</td>
<td>60 ± 32¶</td>
<td>-14.6 (-23.9 to -4.2)</td>
<td>&lt;0.001</td>
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<tr>
<td>P-value§</td>
<td>&lt;0.05</td>
<td></td>
<td></td>
<td></td>
<td>0.26</td>
<td></td>
<td></td>
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<tr>
<td><strong>FVII activity (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.05</td>
<td></td>
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</tr>
<tr>
<td>Intranasal</td>
<td>110 ± 20</td>
<td>93 ± 14¶</td>
<td>94 ± 20¶</td>
<td>94 ± 16¶</td>
<td>-14.0 (-17.0 to -11.1)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Oral</td>
<td>123 ± 32</td>
<td>99 ± 27¶</td>
<td>96 ± 28¶</td>
<td>97 ± 28¶</td>
<td>-20.3 (-24.1 to -16.6)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>P-value§</td>
<td>&lt;0.05</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.01</td>
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<tr>
<td><strong>F1+2 (nmol/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intranasal</td>
<td>0.9 (0.8–1.2)</td>
<td>1.0 (0.8–1.4)</td>
<td>1.0 (0.9–1.3)</td>
<td>1.0 (0.8–1.3)</td>
<td>5.8 (-4.2 to 15.8)</td>
<td>0.99</td>
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</tr>
<tr>
<td>Oral</td>
<td>0.9 (0.7–1.1)</td>
<td>1.0 (0.9–1.3¶)</td>
<td>1.1 (0.8–1.4¶)</td>
<td>1.1 (0.9–1.3¶)</td>
<td>19.0 (5.8 to 32.2)</td>
<td>&lt;0.01</td>
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<tr>
<td>P-value§</td>
<td>0.29</td>
<td></td>
<td></td>
<td></td>
<td>0.11</td>
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<tr>
<td><strong>TAT (μg/L)</strong></td>
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<td></td>
<td></td>
<td></td>
<td>0.38</td>
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<tr>
<td>Intranasal</td>
<td>2.4 (1.8–3.1)</td>
<td>2.1 (1.9–2.7)</td>
<td>2.1 (1.9–2.9)</td>
<td>1.9 (1.7–2.4)</td>
<td>-13.4 (-25.3 to 0.2)</td>
<td>0.19</td>
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<tr>
<td>Oral</td>
<td>2.1 (1.7–2.6)</td>
<td>1.9 (1.7–2.3)</td>
<td>1.9 (1.8–2.5)</td>
<td>2.0 (1.6–2.4)</td>
<td>-5.3 (-19.3 to 11.1)</td>
<td>0.87</td>
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</table>

Values at baseline and at week 12, 24 and 52 as mean ± SD or median (25th–75th percentile), %Δ = percentage change from baseline in week 52 as (geometric) mean (95% confidence interval), or as median (25th–75th percentile). P-value for †unpaired t-test for comparison between the groups or Mann-Whitney U-test for baseline values of homocysteine and for %Δ in D-dimer and PAP, ‡ANOVA for repeated measurements over 52 weeks with the baseline value of the parameter under consideration as covariate for between-group differences in change, and ANCOVA for repeated measurements for within-group changes over the 52 weeks. P<0.05, §P<0.01 and ¶P<0.001 for paired t-test versus baseline. Intranasal, spray containing 175 μg 17β-estradiol (E2) and 275 μg norethisterone; oral, capsule containing 1 mg E2 and 0.5 mg norethisterone acetate; FVIIa, activated Factor VII; FVII act, Factor VII activity; F1+2, prothrombin fragments 1+2; TAT, thrombin-antithrombin complex; tPA, tissue plasminogen activator antigen; PAI-1 ag, plaminogen activator inhibitor-1 antigen; PAI-1 act, PAI-1 activity; PAP, plasmin-α2-antiplasmin complex.
<table>
<thead>
<tr>
<th>Markers of fibrinolysis</th>
<th>Baseline</th>
<th>Week 12</th>
<th>Week 24</th>
<th>Week 52</th>
<th>% ∆ 0–52</th>
<th>ANCOVA*</th>
<th>ANOVA†</th>
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<td><strong>Markers of fibrinolysis</strong></td>
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<td></td>
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<tr>
<td><strong>tPA (μg/L)</strong></td>
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<tr>
<td>Intranasal</td>
<td>4.7 ± 1.7</td>
<td>4.2 ± 1.7</td>
<td>4.1 ± 1.7</td>
<td>4.0 ± 1.5</td>
<td>-10.4 (-17.6 to -3.3)</td>
<td>&lt;0.05</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Oral</td>
<td>5.1 ± 2.4</td>
<td>4.1 ± 1.8</td>
<td>3.8 ± 1.7</td>
<td>4.0 ± 1.7</td>
<td>-17.8 (-25.2 to -10.4)</td>
<td>&lt;0.001</td>
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<tr>
<td>P-value§</td>
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<td></td>
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<td></td>
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<tr>
<td><strong>PAI-1 Ag (μg/L)</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Intranasal</td>
<td>34 (23–54)</td>
<td>30 (21–43)</td>
<td></td>
<td>30 (18–44)</td>
<td>24 (17–48)</td>
<td>-13.8 (-26.5 to 1.2)</td>
<td>0.24</td>
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<tr>
<td>Oral</td>
<td>48 (27–81)</td>
<td>33 (16–50)</td>
<td>30 (18–43)</td>
<td>27 (19–42)</td>
<td>-38.0 (-48.3 to -25.6)</td>
<td>&lt;0.001</td>
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<td></td>
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<td></td>
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<td>&lt;0.01</td>
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<tr>
<td><strong>PAI-1 act (U/mL)</strong></td>
<td></td>
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</tr>
<tr>
<td>Intranasal</td>
<td>11.8 (9.6–16.5)</td>
<td>10.6 (6.4–15.2)</td>
<td></td>
<td>9.9 (7.6–16.7)</td>
<td>9.6 (6.9–14.2)</td>
<td></td>
<td>-17.0 (-31.1 to -0.1)</td>
</tr>
<tr>
<td>Oral</td>
<td>17.1 (9.3–27.5)</td>
<td>11.4 (6.9–17.9)</td>
<td>11.4 (7.2–15.8)</td>
<td>10.4 (6.9–14.6)</td>
<td></td>
<td>-30.6 (-44.1 to -13.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>P-value§</td>
<td>0.16</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><strong>D-dimer (μg/L)</strong></td>
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<tr>
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<td>58 (30–103)</td>
<td></td>
<td>57 (29–89)</td>
<td>16.1 (-21.0–112.9)</td>
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<td>67 (33–133)</td>
<td></td>
<td>53 (23–118)</td>
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<td>17.6 (-28.5–77.2)</td>
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<td>91 (66–120)</td>
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<td>9.1 (7.7–10.7)</td>
<td>9.5 (8.1–11.7)</td>
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<td>2.3 (-2.9 to 7.7)</td>
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<tr>
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<td>9.1 (8.4–10.2)</td>
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</table>
Figure 2. Percentage changes from baseline

FVIIa, activated Factor VII; FVII act, Factor VII activity; F1+2, prothrombin fragments 1+2; TAT, thrombin-antithrombin complex; tPA, tissue plasminogen activator antigen; PAI-1 ag, plaminogen activator inhibitor-1 antigen; PAI-1 act, PAI-1 activity; PAP, plasmin-α2-antiplasmin complex

Percentage change from baseline: *P<0.05, †P<0.01 and ‡P<0.001

Between-group difference: §P<0.05 and ||P<0.01
Discussion

In this randomized, double-blind, 1-year study in healthy postmenopausal women, changes during intranasal E2/NET in coagulation parameters fibrinogen, factor VII activity and prothrombin fragment 1+2 and in fibrinolytic parameters tPA and PAI-1 were less pronounced than the pro-coagulant and pro-fibrinolytic effects seen during oral E2/NETA.

Intranasal administration, compared with oral, requires a lower dose due to its high bioavailability. Previous studies showed 300 μg intranasal E2 to be equivalent to 2 mg oral E2 in climacteric symptom relief11 and in 24 h tissue exposure.15 Equivalent quantities of the prodrug NETA and the active hormone NET provide similar pharmacokinetic profiles for NET concentrations.16 NET was used in the intranasal spray because it is the active hormone and is easy to solute. As until now only three studies11,17,18 reported on the effects of intranasal HT on the markers studied here, transdermal HT is the most comparable route of administration to compare our findings with. An important similarity between these routes is the avoidance of the hepatic first-pass effect.

The differences we found in the effect on coagulation parameters are in line with some studies comparing transdermal and oral HT with respect to fibrinogen,19,20 factor VII activity10,21 and F1+2.9,22 Other studies however, did not find a difference between these administration routes.23,24 None of the studies comparing transdermal and oral HT did find a difference in the effect on FVIIa9 or TAT.10,21 Two previous studies comparing intranasal with oral HT, using unopposed E218 or E2 combined with oral dydrogesterone,11 did not find the difference between the groups to be significant for fibrinogen11,18 and FVIIa.11 The changes we found in the oral group are likely to be real effects, as other studies, including some using oral E2/NETA,25-27 reported similar changes from baseline in fibrinogen,28 FVII activity25,26 and F1+2.9,27 also to be different from placebo.

The reductions in the oral group in FVII and in fibrinogen, suggest an anti-coagulant effect. The increase in F1+2 and no change in TAT, do not confirm this idea. The decrease in FVII activity in the intranasal group was not accompanied by changes in F1+2 or TAT. Our observations suggest minor or no net activation of coagulation during intranasal E2/NET compared to oral E2/NETA.

Smaller changes in tPA an PAI-1 Ag compared with decreases during oral HT, have previously been reported for transdermal HT.9,10 We found no overall, but only a transient difference between intranasal and oral HT on PAP and D-dimer. The lack of an overall difference in these markers is in line with two studies comparing transdermal and oral HT.9,21 Previous studies comparing changes in fibrinolytic parameters during transdermal and oral HT were however inconsistent as others reported no difference in change in tPA and PAI-1 Ag,23,29,30 or a difference in change in PAP and D-dimer.10 Two other studies comparing intranasal and oral HT found changes during intranasal E2 to differ from the increase in tPA and the decrease in PAI-1 Ag during oral E2,18 and found no difference in effect on D-dimer when combined with oral dydrogesterone.11 The changes in tPA and PAI-1 we found in the oral group are in line with placebo-controlled studies finding similar changes also to be different from placebo.9,10,27

In the oral group we observed an increased fibrinolytic activity, since both D-dimer and PAP were higher in the oral group. Changes in the intranasal group were smaller than during oral therapy, suggesting a smaller pro-fibrinolytic effect.

Lower levels of tPA, the initiator of the fibrinolytic process, seem to contradict the observation of a potentiation of endothelial tPA release by estrogen.31 The liver is a production site of the major
regulator of the fibrinolytic system, PAI-1, and is important for the clearance of PAI-1, tPA and tPA/PAI-1 complexes. Hepatic clearance of tPA is faster than that of tPA/PAI-1 complexes. Thus, decreased PAI-1 levels will lead to faster clearance of tPA due to diminished complex formation. We indeed found a moderate though significant correlation between changes in tPA and PAI-1. The difference between the changes seen during oral and intranasal HT on tPA and PAI-1 pleads for an altered hepatic clearance and/or production.

The lack of a difference in change, or even of a change within the groups has been reported for the comparison between transdermal and oral HT. A review of the effects of HT on homocysteine, however, concluded that oral HT lowers homocysteine concentrations. Until now only one study has been published on the effects of intranasal E2 on homocysteine. This study reported a decrease from baseline and compared to placebo after 6 months of therapy.

Based on the results of the Heart and Estrogen/progestin Replacement Study (HERS) and the Women’s Health Initiative (WHI) trial, the lack of a cardioprotective effect of HT may only be associated with oral conjugated equine estrogens plus medroxyprogesterone acetate prescribed to late postmenopausal women. Other estrogen plus progestogen combinations and alternative routes of administration may have other effects, in particular when used by early postmenopausal women.

Transdermal administration appears to be safe with respect to venous thrombosis risk. The effect of transdermal HT on CHD risk has been investigated in the Papworth HRT Atherosclerosis Study (PHASE). This study among women with established CHD found no significant differences between transdermal HT and placebo in cardiac events although the event rate was higher in the HT group. As is the case for the results of HERS and WHI trial, it should be considered that it is plausible that the effects of HT in elderly women in general and women with established atherosclerotic disease in particular, will differ from the effects in healthy women to whom HT is prescribed during the early menopausal years.

The strength of our study is the randomized, double-blind study design and the one-year duration. The study also has limitations. A minimum of six weeks wash-out of previous HT was required prior to inclusion in the study. This period may be too short to allow all haemostatic markers to return to baseline. However, except for five women, all women had in fact a wash-out of more than three months prior to inclusion. We did not include an untreated or placebo control group. However, we compared the effects of the new intranasal spray with an oral reference product. The effects during oral E2/NETA were found to be highly similar to those observed in placebo-controlled studies on the effect of oral E2/NETA.

**Conclusions**

In this study in healthy postmenopausal women, compared to oral E2/NETA therapy, intranasal E2/NET therapy had somewhat less effect on the coagulant / fibrinolytic balance. The clinical relevance of these findings has yet to be established, but they seem to indicate that the choice of route of administration could be of importance for CHD risk.

**Acknowledgements**

We thank all women who participated in the study; DDM Braat, MD, PhD, Radboud University Nijmegen Medical Centre, Nijmegen; MO Verhoeven, MD, and TE Vogelvang, MD, PhD, VU University Medical Center, Amsterdam, for logistical and technical assistance; JJ Emeis, PhD, TNO, Leiden, and
A Kok, Department of Clinical Chemistry, and P Sekeris and C Klopper-Tol, Department of Haematology, VU University Medical Center, Amsterdam, for laboratory analyses.

References


Effects of intranasal versus oral hormone therapy on asymmetric dimethylarginine in healthy postmenopausal women: a randomized study

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

*Atherosclerosis, in press*
Abstract

**Objective:** Oral estrogens reduce asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase, and an independent risk factor for cardiovascular disease. This study was conducted to compare the effect on ADMA between intranasal and oral 17β-estradiol (E2) combined with norethisterone (acetate) (NET(A)) administration in postmenopausal women.

**Methods:** In a two-center, randomized, double-blind, comparative study 90 healthy postmenopausal women (age 56.6 ± 4.7 years) received daily continuous combined intranasal E2/NET 175 μg/275 μg (n=47) or oral E2/NETA 1 mg/0.5 mg (n=43) for one year. At baseline, week 12 and 52, plasma concentrations of ADMA, arginine and symmetric dimethylarginine (SDMA) were measured by high-performance liquid chromatography.

**Results:** Oral E2/NETA reduced ADMA concentrations (7.4%; 95% confidence interval (CI) 10.4 to -4.4%), while intranasal E2/NET had no effect (0.8%; 95% CI -3.7 to 2.1%) after 52 weeks. In both groups, arginine was transiently decreased compared with baseline at week 12 (intranasal: -6.1%; 95% CI -9.1 to -3.0%; oral: 6.5%; 95% CI 10.9 to 2.1%). Only oral E2/NETA reduced SDMA concentrations.

**Conclusions:** Oral administration of E2/NETA reduced ADMA and SDMA concentrations, whereas intranasal administration did not. Both treatments transiently reduced arginine. The decrease in ADMA by oral estrogens could be a key phenomenon in the modulation of nitric oxide synthesis by postmenopausal hormone therapy.
Introduction

For more than a decade observational studies have indicated that postmenopausal hormone therapy (HT) can be protective against coronary heart disease (CHD) in healthy early postmenopausal women. Randomized clinical trial results contrast with the favorable results obtained in earlier observational studies. Whereas in elderly postmenopausal women no benefit or even an early harm was reported for CHD risk with oral HT use, the effect on CHD risk of non-oral routes of administration in younger postmenopausal women is unclear.

Nitric oxide (NO) is a potent vasodilator produced by the endothelium, and diminished NO availability has been postulated to play a role in the development of CHD. Asymmetric dimethylarginine (ADMA), an endogenously produced methylated form of arginine, inhibits NO synthesis. High levels of ADMA have been associated with increased cardiovascular event risk and mortality in specific patient groups. In women, a negative correlation between endogenous estradiol and ADMA concentrations has been reported. Both oral and transdermal administered HT reduce ADMA concentrations, with larger reductions after oral than after transdermal administration.

In addition to the oral and transdermal route of administration, a spray has become available for intranasal administration of 17β-estradiol (E2) (Aerodiol®, Servier, Courbevoie, France). This form of administration has shown to be a well-tolerated, effective alternative route for HT. By avoiding the hepatic first-pass effect, less intra- and inter-subject variability in E2-exposure was observed. As successor of the E2-only spray, a new intranasal spray for continuous combined 17β-estradiol and norethisterone (E2/NET) administration has been developed. As hepatic metabolism is largely bypassed, it is plausible that, just as with transdermal patches, the intranasal E2/NET spray would have limited effect on ADMA concentrations. The assumption that intranasal E2 avoids the first-pass effect on the liver is based on the observations that intranasal administration has less effects on sex hormone binding globuline, the lipid profile and C-reactive protein (CRP).

Arginine is a precursor of NO, whereas symmetric dimethylarginine (SDMA) is a stereo-isomer of ADMA that does not inhibit NO-synthase (NOS). The ratio between arginine and ADMA is considered an important parameter for NOS activity. In rabbits, supplementation of arginine can reduce the inhibiting effect of ADMA on NOS and partly restore NO production. This is why the arginine/ADMA ratio was calculated in this study as well.

We hypothesized that the reduction in ADMA concentrations after intranasal E2/NET would be less than after oral E2/NETA, since it is assumed that intranasal E2/NET has less effect on the liver. Therefore, we performed this study comparing the effect of intranasal E2/NET formulation with oral low-dose continuous combined E2/NETA on ADMA, arginine, SDMA concentrations and the arginine/ADMA ratio. This study was nested within a large international, randomized, double-blind, double-dummy study, including 954 women in total with endometrial-safety as primary endpoint, as a substudy (n=90) among participants in two Dutch centers. This is the latest substudy in a series of publications generated from this large international study.
Materials and Methods

Participants

This substudy was performed as part of a large international, randomized, double-blind, double dummy study with two parallel treatment arms in which 50 centers participated (Australia, Argentina, Czech Republic, Italy, Mexico, The Netherlands and Sweden). For logistic reasons it was decided to include only the women from two centers in the Netherlands for this substudy.

Healthy postmenopausal women, aged between 40 and 75 years, were recruited from outpatient clinics and through advertisements in regional newspapers. All women were non-hysterectomized and had their last menstrual period at least two years before inclusion. Serum endogenous estradiol concentrations had to be <110 pmol/L and follicle-stimulating hormone (FSH) >30 IU/L. All participants had a normal cervical smear and mammography within 12 months before inclusion, and a trans-vaginal ultrasound and blood tests (lipids, liver enzymes, kidney function, glucose and thyroid stimulating hormone) without any clinically relevant abnormalities. At screening all participants had plasma concentrations of total cholesterol of ≤8.0 mmol/L and triglycerides ≤3.0 mmol/L.

Exclusion criteria were a body mass index (BMI) >32 kg/m², any contraindication for use of estrogen and/or progestogen, and any ear-nose-throat disease that might interfere with intranasal drug administration. During the study, women were prohibited from using: any treatment for menopausal symptoms, chronic treatment liable to interfere with the coagulation profile, treatment liable to interfere with intranasal drug administration, enzyme inducers and systemic vasoconstrictors. This study was done in a subset of women without a history of HT use, or who had a wash-out of at least 6 weeks before the baseline visit, and who were not taking lipid-lowering drugs, since these treatments can influence ADMA concentrations.

All participants gave written informed consent before participation in the trial, which was conducted in accordance with the ethical principles stated in the Declaration of Helsinki, with Good Clinical Practice, and with approval of the central and local institutional review boards.

Study design

After a 1 to 6 week screening period, eligible women were randomized to daily either one intranasal spray containing a fixed dose of 175 μg 17β-estradiol combined with 275 μg norethisterone (E₂/NET) (521405, Servier, Courbevoie, France) and one placebo capsule (intranasal group) or one capsule containing 1 mg 17β-estradiol combined with 0.5 mg norethisterone acetate (E₂/NETA) (Activelle®, Novo Nordisk, Bagsvaerd, Denmark) and one placebo spray (oral group). Study medication was manufactured, packaged and labeled by the Institut de Recherches Internationales Servier (I.R.I.S.; Courbevoie, France). Placebos and active treatments were identical in appearance and smell.

The rationale for the dosages chosen was that the 24-hour exposure after 300 μg intranasal E₂ was similar to that of 2 mg oral E₂.¹⁹ Intranasal 548 μg NET provided a NET exposure similar to oral 1 mg NET. Equivalent quantities of NETA and NET provide similar pharmacokinetic profiles for NET concentrations.²⁰ Comparing intranasal E₂ alone with intranasal E₂ combined with NET showed a decrease of the bioavailability of estradiol. It was shown that the exogenous estradiol exposure of 345 μg E₂ combined with NET was equivalent to the exogenous estradiol exposure after 300 μg E₂ alone. In this study, in line with the current recommendation to use low-dose HT, half the dosage of
345 μg intranasal E2 was administered, corresponding with 1 mg oral E2 and half the dosage of 548 μg of NET was administered corresponding with 0.5 mg NETA.20

Centralized computerized subject randomization was done by an Interactive Voice Response System in blocks of 12 (6 active spray and 6 active capsules) per center. Treatment was administered for 52 weeks. Throughout the whole study period, all participants, clinical investigators, and laboratory personnel were blinded for the study medication. Unblinding was done after all data were collected in the database.

**Blood sampling**

For assessment of ADMA, arginine and SDMA concentrations, venous blood samples were taken at baseline and in week 12 and 52 between 8:00 and 10:00 a.m. 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 into cooled tubes containing tri-potassium ethylenediaminetetra-acetic acid (K₃EDTA) (Becton Dickinson, Meylan, Cedex-France). After blood collection, tubes were immediately placed in ice. Within one hour after collection, plasma was separated by centrifugation at 2000 g for 30 minutes at 4°C. Plasma was divided into aliquots, snap-frozen and stored at -80°C until analysis.

ADMA, arginine, and SDMA were measured by high-performance liquid chromatography with fluorescence detection.21 All samples from individual patients were analyzed in the same analytical series. The inter-assay coefficients of variation were less than 3% for ADMA and arginine and less than 4% for SDMA. Plasma arginine/ADMA ratios were calculated for each participant at each visit.

**Statistical analyses**

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) 10.0.5 for Windows (SPSS Inc., Chicago, IL, USA). Values of the investigated parameters are given as mean ± standard deviation or as median (25th–75th percentile) if skewed. Percentage changes from baseline are given as mean (95% confidence interval (CI)) or as geometric mean (95% CI) if the percentage changes were skewed.

Ad hoc statistical analyses were performed using standard parametric tests; if the distribution of variables was skewed, analysis was done after log-transformation. Baseline values were compared using an unpaired t-test or a χ²-test where applicable. For between-group comparisons we used analyses of covariance (ANCOVA) for repeated measurements, with the baseline value of the variable under consideration as constant covariate. Within-group changes over time were tested using analysis of variance (ANOVA) for repeated measurements and paired t-tests versus baseline. Percentage changes where compared with unpaired t-tests.

Only data from women with data available at baseline and at week 12 were used for the analyses. In the oral group, in one woman, who discontinued the study earlier than planned, an additional blood sample was taken. We analyzed this blood sample as if taken at the last scheduled visit. Therefore, the last-observation-carried-forward procedure was applied in seven cases (intranasal n=1; oral n=6) for missing values using the value of the visit at week 12 for AN(C)OVA for repeated measurements.

After finding clinical interesting results it was decided to investigate post hoc associations of age, BMI, time since menopause, blood pressure, FSH, and endogenous E₂ with baseline concentrations
of ADMA by calculating Pearson’s correlation coefficient. Previously, we reported on the following CHD risk markers measured in this trial: total cholesterol, low-density lipoprotein (LDL)-cholesterol, high-density lipoprotein (HDL)-cholesterol, HDL_2- and HDL_3-cholesterol, triglycerides, CRP, soluble vascular cell adhesion molecule-1 (sVCAM-1), soluble intercellular adhesion molecule-1 (sICAM-1) and soluble E-selectin (sE-selectin). Pearson’s correlation coefficients were calculated between baseline values of ADMA and baseline values of arginine, SDMA, and the CHD risk markers, measured previously in this study and between the absolute changes in ADMA and arginine, SDMA and these risk markers. For these post-hoc analyses the Bonferroni p-correction for multiple comparisons was applied. The accepted significance level here was: P=0.05/32=0.002.

**Results**

Between September 2001 and June 2002 a total of 125 women were screened in two Dutch centers, of whom 94 women were randomized. Four women either had no wash-out from their previous HT (n=3) or used lipid-lowering drugs (n=1). These women were excluded from this substudy. Ninety women (intranasal n=47; oral n=43) were found eligible for the current substudy (Figure 1). At baseline, no significant differences were found between the groups in either demographic characteristics or in any of the variables investigated (Table I). The last patients completed the study in May 2003.

Two women in the intranasal group discontinued the study compared to ten in the oral group (P<0.01) (Figure 1). Premature study discontinuation was mainly related to the occurrence of an adverse event. No women stopped because of the occurrence of a coronary or a cerebrovascular event. Two women discontinued early because of symptoms suspicious for deep venous thrombosis

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**Table I. Characteristics of the two groups at baseline**

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<tr>
<td>Age (years)</td>
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<td>Body mass index (kg/m2)</td>
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<td>11 (25.6%)</td>
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<td>Serum cholesterol (mmol/L)</td>
<td>6.1 ± 1.1</td>
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<tr>
<td>Serum endogenous estradiol (pmol/L)</td>
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Values are given as mean ± standard deviation, as median (25th–75th percentile) or as number (n) with percentage in parentheses. There were no statistically significant differences between the two groups in the baseline characteristics. FSH, follicle-stimulating hormone; intranasal, spray containing 175 μg 17β-estradiol (E2) and 275 μg norethisterone; oral, capsule containing 1 mg E2 and 0.5 mg norethisterone acetate.
One DVT was confirmed (intranasal group) while the other could not be confirmed ultrasonographically (oral group). Furthermore, one woman in the oral group stopped because of the detection of breast cancer and one woman in the intranasal group was excluded from analyses after week 12 because of the start of preventive anticoagulant therapy because of a family history of cerebrovascular disease. As for four women only baseline data were available, analyses were based on 86 women (intranasal n=46; oral n=40) of whom values at baseline and at week 12 were available.

Table II provides plasma concentrations of ADMA, arginine and SDMA and the calculated arginine/ADMA ratio at baseline and after 12 and 52 weeks of treatment. After 52 weeks, no effect of intranasal E₂/NET on ADMA concentrations was observed. The significant reduction in ADMA concentrations in the oral E₂/NETA group was already present at week 12 (-6.3%; 95% CI -9.3 to -3.2%) and sustained in week 52 (-7.4%; 95% CI -10.4 to -4.4%). The mean percentage decrease in ADMA concentrations found in the oral group differed significantly from that in the intranasal group at week 12 and 52 (P=0.02 and P<0.01 respectively; Figure 2).

Figure 1. Clinical trial profile.
The numbers of women in the substudy in which ADMA, arginine and SDMA were measured at baseline, week 12 and week 52.
intranasal, spray containing 175 μg 17β-estradiol (E₂) combined with 275 μg norethisterone; oral, capsule containing 1 mg E₂ combined with 0.5 mg norethisterone acetate.
Both intranasal and oral administration revealed a transient decrease (of approximately 6%) in arginine in week 12, which had disappeared in week 52. No significant difference in effects on arginine was found between the intranasal and oral groups. Intranasal E₂/NET did not induce any effects on SDMA during 52 weeks of treatment (Table II). The ANOVA for within-group changes in SDMA in the oral group was significant (P=0.03). The mean percentage reductions were -3.4% (95% CI -6.9 to 0.0%) at week 12 and -4.0% (95% CI -8.5 to 0.5%) at week 52. The non-significant decrease of the arginine/ADMA ratio in the intranasal group of -4.8% (95% CI -10.1 to 1.5%) and

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<th>ANOVA‡</th>
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<td>Intranasal</td>
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<td>0.439 ± 0.042</td>
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<td>Oral</td>
<td>0.462 ± 0.070</td>
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<td>0.431 ± 0.063**</td>
<td>&lt; 0.001</td>
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<td></td>
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<td>&lt; 0.01</td>
</tr>
<tr>
<td><strong>Arginine (μmol/l)</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Intranasal</td>
<td>110.7 ± 19.1</td>
<td>107.9 ± 18.7</td>
<td>107.9 ± 18.7</td>
<td>&lt; 0.01</td>
<td>-6.1 (-9.1 to -3.0)</td>
<td>-1.6 (-7.6 to 4.3)</td>
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</tr>
<tr>
<td>Oral</td>
<td>107.0 ± 17.5</td>
<td>105.3 ± 16.0</td>
<td>105.3 ± 16.0</td>
<td>0.03</td>
<td>-6.5 (-10.9 to -2.1)</td>
<td>-1.7 (-6.4 to 3.0)</td>
<td></td>
</tr>
<tr>
<td>P-value‖</td>
<td>0.36</td>
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<tr>
<td></td>
<td>0.87</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.99</td>
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<tr>
<td><strong>SDMA (μmol/L)</strong></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Intranasal</td>
<td>0.501 ± 0.066</td>
<td>0.493 ± 0.065</td>
<td>0.493 ± 0.065</td>
<td>0.23</td>
<td>-2.2 (-5.3 to 1.0)</td>
<td>-0.9 (-4.2 to 2.3)</td>
<td></td>
</tr>
<tr>
<td>Oral</td>
<td>0.493 ± 0.076</td>
<td>0.478 ± 0.063*</td>
<td>0.478 ± 0.063*</td>
<td>0.03</td>
<td>-3.4 (-6.9 to 0.0)</td>
<td>-4.0 (-8.5 to 0.5)</td>
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<tr>
<td>P-value‖</td>
<td>0.61</td>
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<td></td>
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<tr>
<td></td>
<td>0.58</td>
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<td></td>
<td></td>
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<td></td>
<td>0.26</td>
</tr>
<tr>
<td><strong>Arginine/ADMA ratio¶</strong></td>
<td></td>
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</tr>
<tr>
<td>Intranasal</td>
<td>246 (221-276)</td>
<td>236 (220-264)</td>
<td>236 (220-264)</td>
<td>0.13</td>
<td>-6.4 (-10.0 to -2.5)</td>
<td>-4.8 (-10.1 to 1.5)</td>
<td></td>
</tr>
<tr>
<td>Oral</td>
<td>226 (207-260)</td>
<td>247 (215-268)*</td>
<td>247 (215-268)*</td>
<td>0.03</td>
<td>-2.4 (-6.8 to 2.7)</td>
<td>4.5 (-0.0 to 9.6)</td>
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<tr>
<td>P-value‖</td>
<td>0.10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.03</td>
</tr>
</tbody>
</table>

Concentrations are given as mean ± standard deviation and ratios are given as median (25th - 75th percentile).

ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine; intranasal, spray containing of 175 μg 17β-estradiol (E₂) combined with 275 μg norethisterone; oral, capsule containing 1 mg E₂ combined with 0.5 mg norethisterone acetate.

†Analysis of covariance for repeated measurements (ANCOVA) for between-group differences with the baseline value of the variable under consideration as constant covariate, over the 52-week study period.

‡Analysis of variance (ANOVA) for within-group changes over time.

§% change: Mean (95% confidence interval (CI)) of the individual percentage changes from baseline at week 12 and at week 52.

‖Unpaired T-test for difference between the groups.

*P<0.05; **P≤0.01 Paired T-test for within-group change from baseline at the different time points.

¶Arginine/ADMA ratio was not normally distributed and therefore log-transformed before analyses. Percentage changes are given as geometric mean (95% CI).
increase in the oral group of 4.5% (95% CI -0.02 to 9.6), resulted in a significant between-group difference at week 52 (P=0.03).

None of the baseline characteristics correlated with baseline ADMA concentrations. At baseline, ADMA concentrations were significantly correlated with SDMA concentrations (r=0.42; P<0.001) and sE-selectin levels (r=0.39; P<0.001). The absolute changes in ADMA at week 52 were correlated with the changes in SDMA (r=0.59; P<0.001) and with the changes in sE-selectin (r=0.45; P<0.001). None of the other CHD markers that were measured previously in this study (total cholesterol, LDL-cholesterol, HDL-, HDL2- and HDL3-cholesterol, triglycerides, CRP, sVCAM-1 and siCAM-1) correlated significantly with ADMA concentrations, neither in their baseline values nor in changes from baseline at week 52.

**Figure 2.** Mean percentage change from baseline in ADMA, arginine, SDMA after 52 weeks. Error bars represent standard error of the mean.

ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine; intranasal, spray containing of 175 μg 17β-estradiol (E2) combined with 275 μg norethisterone; oral, capsule containing 1 mg E2 combined with 0.5 mg norethisterone acetate.

*P<0.01: unpaired t-test for between group differences.
†P<0.001: one-sample t-test for percentage changes.
Discussion

One year of intranasal administration of 17β-estradiol combined with norethisterone had no effect on plasma ADMA concentrations in healthy postmenopausal women. In contrast, oral administration of 17β-estradiol combined with norethisterone acetate significantly reduced the mean ADMA concentration at week 12 which persisted during the following 40 weeks. Arginine was transiently reduced in both the intranasal and the oral group, resulting in no significant change at week 52 in both groups. The absolute SDMA concentrations showed a significant reduction in the oral group only.

In three previous studies and in the present study, ADMA concentrations were significantly reduced by oral estrogens and to a similar extent (approximately 8%), indicating that the moderate ADMA-lowering effect of oral estrogens is not a spurious finding but a real phenomenon.\textsuperscript{10-12} Although the absolute effect may seem small, it is noteworthy that the biological variation of plasma ADMA concentrations is also very small, with an inter-individual coefficient of variation in the general population of approximately 12%.\textsuperscript{22} The treatment-induced reduction of 8% thus equals approximately two-thirds of the standard deviation, which is considered a moderate effect size. In addition, it has been shown that even slightly increased ADMA concentrations are independently associated with cardiovascular events.\textsuperscript{6,8} Therefore, in our opinion the moderate reductions in ADMA levels, as observed in the present and previous studies, may be of clinical relevance.

Both ADMA and SDMA are formed by methylation of arginine residues in proteins and free ADMA and SDMA, as measured in this study, are released upon proteolysis of these proteins. SDMA is mainly cleared by renal excretion, whereas only a small part of ADMA is cleared from the circulation by this pathway.\textsuperscript{23} 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, which was not found.

Approximately 80% of ADMA is metabolized by the widely expressed enzyme dimethylarginine dimethylaminohydrolase (DDAH).\textsuperscript{23} DDAH is very sensitive to oxidative stress and pathological stimuli that induce oxidative stress have been shown to reduce DDAH activity and lead to an accumulation of ADMA. Conversely, compounds with anti-oxidant properties, possibly including estrogens, may protect DDAH from inactivation by oxidants, leading to a reduction of ADMA concentrations. It may be that estrogen has a direct effect on DDAH activity or that HT lowers ADMA concentrations by other mechanisms, such as a diminished methylation of arginine residues in proteins or a reduction of proteolysis.

The liver plays an important role in the elimination of ADMA, probably through the degradation of ADMA by DDAH.\textsuperscript{24-26} This may provide an explanation for the smaller reduction after transdermal HT administration (4%) than after oral administration (approximately 8%) described earlier.\textsuperscript{12} Unlike oral estrogens, transdermally and intranasally administered estrogens directly enter the systemic circulation without a first-pass through the liver. Therefore it was expected that intranasal, just like transdermal administration, would have less effect on the ADMA concentration than oral administration. However, this does not explain why transdermal administration significantly reduced the ADMA concentration compared with baseline, whereas intranasal administration had absolutely no effect. The difference in pharmacokinetics between pulsed intranasal and continuous transdermal administration possibly provides an explanation.\textsuperscript{19,27} After intranasal administration, estradiol is
rapidly absorbed and induces a very steep and short-lived peak in plasma levels whereas transdermal administration causes a prolonged estrogen exposure. Possibly, a prolonged exposure to a minimum level of estradiol is needed for an adequate reduction of ADMA concentrations, and the transient estradiol peak after intranasal administration is not effective in this respect.

An alternative explanation, also related to the difference in pharmacokinetics, is that the total exposure to estrogens could be lower for the intranasal compared to the transdermal or oral route of administration. The 24 hour exposure after 300 μg intranasal E2 was similar to that of 50 μg transdermal and 2 mg oral. The dosage of the transdermal therapy used in the previous study, which found a 4% reduction in ADMA concentrations by transdermal E2, was 50 μg daily. In this study, in line with the current recommendation to use low-dose HT, half the dosage of E2 was administered intranasally and this would correspond with 25 μg E2 transdermally. Therefore, the dosage used in the intranasal administration may possibly have been too low to affect ADMA concentrations.

Another difference between the intranasal administration in this study and the transdermal administration in the previous study is the addition of NET to the intranasal route. NET is a 19-nortestosterone derivative with a partial androgenic activity, which can reverse estrogen-induced effects independent of its route of administration. The average reduction in ADMA induced by oral E2/NETA in this study (approximately 7%) is similar to the reductions after E2 alone or E2 combined with gestodene and dydrogesterone. Gestodene and dydrogesterone did not modify the effect of oral E2 on ADMA concentrations. From these earlier observations and the results of the present study it is plausible that NET does not modify the oral E2 induced ADMA reductions and that it is unlikely that NET would have modulated the effect of intranasal E2.

The studies describing high ADMA concentrations in patients with CHD or high CHD risk included mostly men. The evidence for a relation between CHD and high ADMA concentrations in women is much less clear. In a group of women with CHD (mean age 58 years), ADMA concentrations were significantly higher compared with women without CHD (mean age 54 years). Therefore, the association between high ADMA concentrations and high CHD risk is probable also true in women.

Up till now, no longitudinal data of ADMA concentrations in women during the menopausal transition have been published. One study observed an inverse relation between ADMA concentrations and endogenous estradiol concentrations in women. Schulze et al. observed a significantly higher ADMA concentration in women over 50 years than in younger women. Although menopausal state and HT use were not documented in the study, this age-related difference, which was not observed in men, suggest that ADMA levels may increase with the onset of menopause. The consistent observations of the reduction of ADMA by HT in several studies make it plausible to expect that ADMA concentrations would increase in women undergoing the menopausal transition due to decreasing endogenous estrogen concentrations. The present study does not support this assumption, given that there was no significant correlation between ADMA concentrations and endogenous estradiol concentrations, FSH concentrations and time since menopause.

At baseline, ADMA showed a significant positive association with the adhesion molecule sE-selectin. In addition, a positive association was found between treatment-induced changes in ADMA and sE-selectin. An association between ADMA and sVCAM has been described before whereas, an association between ADMA and sE-selectin has not been studied up till now. One of the
mechanisms by which NO exerts its anti-atherogenic effect is inhibition of endothelial adhesiveness. Since ADMA inhibits NO production, it is possible that ADMA influences endothelial adhesiveness. Increased endothelial adhesiveness in the presence of elevated ADMA concentrations has indeed been demonstrated in cultured endothelial cells and in hypercholesterolemic humans.

A major strength of this study is the randomized 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. A potential limitation of this study is the absence of a placebo group. However, we compared the effects of the new intranasal spray with a widely studied oral reference product. The effects found in the oral group in this study were comparable with those observed in other placebo-controlled studies. The sample size calculation was based on changes in normalized APC sensitivity ratio. For the effects on ADMA concentrations the observed power was 93%. However, for the changes in arginine, SDMA and arginine/ADMA ratio the observed power was 5%, 30% and 44%, respectively. Conclusions from non-significant changes in these last three markers are therefore difficult to draw.

To conclude, ADMA concentrations were reduced by oral E₂/NETA, and not by intranasal E₂/NET therapy. Although high levels of ADMA are associated with an increase in CHD events, the clinical implications of ADMA reductions by oral HT are at present unclear and need further investigation.

Acknowledgments

The authors wish to thank Mrs TE Vogelvang, MD, PhD, VU University Medical Center, Amsterdam, The Netherlands, for excellent logistical assistance and recruitment of participants, and the following investigators who participated in this study: Mrs DDM Braat, MD, PhD, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands; Mrs C Klipping, MD, Dinox Medical Investigations, Nijmegen, The Netherlands; and Mrs S de Jong, department of Clinical Chemistry, VU University Medical Center, Amsterdam, The Netherlands, for performing laboratory analyses, and all women who participated in the study.

This study was supported by a research grant from the Institut de Recherches Internationales Servier (I.R.I.S.) to the Biocare foundation (grant no 01-049).

References


