Menopause,
menopausal therapies
and
asymmetric dimethylarginine,
an emerging cardiovascular risk factor

Marieke O. Verhoeven
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dr. T. Teerlink
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CHAPTER 1

General introduction and outline of the thesis

The menopause can be defined as the point in time of the last vaginal bleeding from the endometrium induced by the influence of hormones produced by the ovaries.⁠¹ Women experience a physiological menopause on average at the age of 51 years.⁠² After the menopause the production of female sex hormones in the ovaries reduces significantly. During the menopausal transition, approximately 85% of women experience vasomotor symptoms and genital atrophy for some time. One third of these women describe their symptoms as severe and will seek medical help.⁠³ Oestrogen alone or combined with a progestogen is the first choice of treatment for women with disabling perimenopausal symptoms.⁠¹ Both treatment options will be referred to here as postmenopausal hormone therapy (HT).

Arterial disease is the leading cause of death in men and women in Western countries. Before the age of 60 years the incidence of arterial disease in women lags approximately 10 years behind that of men.⁠⁴ After the menopause the incidence in arterial disease rapidly increases.⁠⁴ Both men and postmenopausal women have a higher arterial disease incidence than premenopausal women and an early menopause is associated with a higher arterial disease risk.⁠⁴–⁶ The postmenopausal status is particularly characterised by low endogenous oestrogen concentrations. These observations resulted in the hypothesis that endogenous oestrogen protects premenopausal women from arterial disease and that HT could prevent the acceleration of arterial disease risk in postmenopausal women.

Although observational studies confirmed this hypothesis,⁷,⁸ large randomised clinical trials (RCTs) failed to show this protective effect and furthermore, demonstrated health risks, such as venous thromboembolism and stroke after oral HT.⁹,¹⁰ The women in these RCTs were on average 63 years of age and therefore approximately on average 10 years postmenopausal. So, these women had been oestrogen depleted for 10 years, which is associated with unfavourable changes in the vascular system.¹¹ Possibly, the harmful effects of HT on some processes associated with cardiovascular health (for example coagulation¹² and inflammation¹³,¹⁴) outweigh the beneficial effects of HT on other processes associated with cardiovascular health (for example lipid metabolism¹⁵ and flow mediated dilatation¹⁶) in late postmenopausal women. In contrast, in early postmenopausal women the effect of HT on cardiovascular health remains largely unknown. It has been postulated that treatment of women with HT early after menopause may prevent the accelerated deterioration of the vascular system.¹¹ The relatively healthy vascular system together with the beneficial effects of HT on several processes associated with cardiovascular health outweighs the harmful effects of HT on other processes associated with cardiovascular health resulting in an overall reduced arterial disease risk. This hypothesis thus
warrants RCTs into the effect of HT in early postmenopausal women on arterial
disease risk markers and on clinical arterial disease outcomes.

In the previously mentioned RCTs, women were given oral HT in only one
dosage. Consequently, the results of these trials cannot automatically be extra-
polated to other administration forms like transdermal or intranasal or to other
dosages (more particular: low dosages). Therefore, it is important to explore alter-
native administration routes beside the oral route for HT, as well as the effect of
different dosages and especially lower dosages of HT. In addition, it is important
to investigate alternative treatment options for HT, like plant derived substances
(for example soy isoflavones or Actaea racemosa linnaeus, (Actaea racemosa L.)) or
selective oestrogen receptor modulators (SERMs), with a benefit/risk profile that
is more acceptable for the treatment of symptomatic menopausal women.

1. Oestrogens and nitric oxide

Nitric oxide (NO) plays a critical role in the cardiovascular system, the nervous
system and in inflammation processes and in the immunological defence system. NO is produced by NO-synthase (NOS) which is mainly expressed by endothe-
lial cells, neurons and macrophages. For this thesis the role of NO in the vascular system is important. NO induces vasodilatation and inhibits platelet aggregation, leukocyte-vessel wall interaction and smooth muscle cell proliferation, all processes involved in the atherosclerotic process, which is the underlying pathology of arterial disease. The biological precursor is arginine from which NOS generates NO and citrulline.

Oestrogens influence the production of NO in several ways. They increase NO synthesis through stimulation of NOS expression and activity. The enhancement of NOS expression by oestrogens is a long-term genomic effect whereas the increase in activity of NOS by oestrogens is a rapid non-genomic effect and both effects are oestrogen-receptor dependent. Multiple signal transduction events are likely involved in the oestrogen-induced increase in NOS-activity.

Endogenously produced NOS inhibitors reduce NO synthesis by preventing arginine to bind to NOS. The reversible binding to NOS of these NOS inhibitors can be disrupted with an abundance of arginine. Asymmetric dimethylarginine (ADMA) is an important endogenous NOS inhibitor. This thesis will explore the effect of oestrogens on the endogenous NOS inhibitor ADMA in middle-aged healthy women.

2. Asymmetric dimethylarginine

Asymmetric dimethylarginine is an end product of the proteolysis of proteins containing methylated arginine, for example heat shock proteins, nuclear and nu-
clidean proteins (Figure 1). In 1992 it was discovered that ADMA is an endogenous NOS inhibitor and that it reduces NO release by macrophages. Furthermore, local infusion of this component into the brachial artery of healthy volunteers caused a dose-dependent fall in forearm blood-flow. ADMA is removed from the body by being metabolised to citrulline by the widely expressed enzyme dimethylarginine dimethylaminohydrolase (DDAH) and by renal excretion, which is the net result of glomerular filtration, tubular secretion and tubular reabsorption. The net extraction of ADMA by the kidney is the sum of urinary excretion and degradation by renal DDAH (Figure 2).
<p>| Arterial disease and its risk determinants associated with elevated ADMA blood concentrations |
|---------------------------------|----------------|---------------------------------|</p>
<table>
<thead>
<tr>
<th><strong>arterial disease</strong></th>
<th><strong>references</strong></th>
<th><strong>risk determinant</strong></th>
<th><strong>references</strong></th>
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</thead>
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<td>Renal diseases</td>
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<td>45-47</td>
<td>Diabetes mellitus(b)</td>
<td>74,75</td>
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<tr>
<td>Myocardial infarction</td>
<td>37</td>
<td>Type 1</td>
<td>72</td>
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<tr>
<td>Unstable angina pectoris</td>
<td>37,39</td>
<td>Type 2</td>
<td>76-79</td>
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<tr>
<td>Stable angina pectoris</td>
<td>39</td>
<td>Systemic lupus erythematosus</td>
<td>80</td>
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<td>Vasospastic angina</td>
<td>48</td>
<td>Hypercholesterolaemia(c)</td>
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<td>Cardiac syndrome X</td>
<td>49,50</td>
<td>Hyperhomocysteinaemia(d)</td>
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<tr>
<td>Stroke</td>
<td>51</td>
<td>Hypertriglyceridaemia(e)</td>
<td>101-103</td>
</tr>
<tr>
<td>Atherosclerosis:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coronary artery(a)</td>
<td>52,53</td>
<td>Sex(f)</td>
<td>75,105</td>
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<tr>
<td>Carotid artery</td>
<td>54,55</td>
<td>BMI(g)</td>
<td>102,106</td>
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<td>Peripheral artery</td>
<td>56-59</td>
<td>Obesity</td>
<td>107</td>
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<td>Essential hypertension</td>
<td>60-63</td>
<td>Smoking(h)</td>
<td>38,98,106,108,109</td>
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<tr>
<td>Pulmonary arterial hypertension</td>
<td>65-66</td>
<td>Cytomegalovirus infection</td>
<td>110</td>
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<tr>
<td>White coat hypertension</td>
<td>63</td>
<td>Adhesion molecules</td>
<td>84,111-115</td>
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<td></td>
<td>Angiogenesis</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CRP(i)</td>
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<tr>
<td></td>
<td></td>
<td>FMD(j)</td>
<td>30,82,116</td>
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<td></td>
<td></td>
<td>IMT(k)</td>
<td>54,55,58,74,113</td>
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<tr>
<td></td>
<td></td>
<td>Von Willebrand Factor</td>
<td>114</td>
</tr>
</tbody>
</table>

Table 1: BMI, body mass index; CRP, C-reactive protein; FMD, flow mediated dilatation; IMT, intima-media thickness.
\(a\) Two studies did not find a difference between a population with coronary stenotic lesions versus a control group.\(^{98,117}\)
\(b\) One study found lower ADMA concentrations in diabetic patients.\(^{103}\)
\(c\) Three studies did not find a correlation between ADMA and LDL-cholesterol\(^{103,114,118}\).
\(d\) Several studies found no relation between ADMA concentrations and hyperhomocysteinaemia.\(^{100,119-121}\)
\(e\) Two studies found no correlation between ADMA and triglycerides concentrations.\(^{99,122}\)
\(f\) No correlation between ADMA concentrations and triglycerides concentrations.\(^{37,95,97}\)
\(g\) No correlation was found between ADMA concentrations and BMI in one study.\(^{99}\)
\(h\) Some studies did not find a difference in ADMA concentrations between smokers and non-smokers\(^{37,123}\) and two studies found a reduced ADMA concentration in smokers.\(^{97,99}\)
\(i\) No association between ADMA and CRP concentrations was found in three studies.\(^{39,99,113}\)
\(j,k\) No correlations between ADMA concentrations and FMD and IMT were observed in another study.\(^{117}\)
Figure 1. Molecular structures of arginine, MMA, ADMA, and SDMA. ADMA, asymmetric dimethylarginine; MMA, monomethylarginine; SDMA, symmetric dimethylarginine.

Other diseases associated with elevated ADMA blood concentrations

<table>
<thead>
<tr>
<th>disease / condition</th>
<th>references</th>
</tr>
</thead>
<tbody>
<tr>
<td>blood diseases</td>
<td>TTP / HUS</td>
</tr>
<tr>
<td></td>
<td>sickle cell disease</td>
</tr>
<tr>
<td>central nervous diseases</td>
<td>Alzheimer’s disease&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>schizophrenia</td>
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<tr>
<td>endocrine diseases</td>
<td>hyperthyroidism</td>
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<td></td>
<td>hypopituitarism</td>
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<tr>
<td></td>
<td>idiopathic hypogonadism</td>
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<tr>
<td>intensive care unit</td>
<td>critically ill patients</td>
</tr>
<tr>
<td></td>
<td>mortality</td>
</tr>
<tr>
<td></td>
<td>septic shock</td>
</tr>
<tr>
<td>muscular diseases</td>
<td>Duchenne muscular dystrophy&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>limb-girdle muscular dystrophy&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>organ transplantation</td>
<td>heart</td>
</tr>
<tr>
<td></td>
<td>liver with poor outcome</td>
</tr>
<tr>
<td>pregnancy complications</td>
<td>intra-uterine growth retardation</td>
</tr>
<tr>
<td></td>
<td>HELLP syndrome</td>
</tr>
<tr>
<td></td>
<td>pre-eclampsia&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>previous gestational diabetes</td>
</tr>
<tr>
<td>others</td>
<td>haemorrhagic shock</td>
</tr>
<tr>
<td></td>
<td>low bone mineral density</td>
</tr>
</tbody>
</table>

Table 2: HELLP, haemolysis, elevated liver enzymes and low platelets; HUS, haemolytic uraemic syndrome; TTP, thrombotic thrombocytopenic purpura.

<sup>a</sup> Another study found no difference in ADMA concentrations in cerebrospinal fluid in patients with Alzheimer’s disease compared with controls. 143

<sup>b</sup> ADMA concentrations were measured in urine. 131

<sup>c</sup> Two studies found no difference between pregnant women with and without pre-eclampsia. 135,144
Arterial disease and several arterial disease risk determinants are associated with elevated ADMA blood concentrations (Table 1). Besides these observations, high ADMA concentrations are associated with future arterial disease as well.\textsuperscript{34-40} Although the link between ADMA and arterial disease has been established most firmly, many other clinical conditions and diseases are associated with elevated ADMA concentrations as well (Table 2).\textsuperscript{41} Also influences of life style habits (Table 3) and of therapeutic agents (Table 4) on ADMA concentrations have been described in the literature.

Symmetric dimethylarginine (SDMA), a stereoisomer of ADMA, does not inhibit NOS activity (Figure 1). Both ADMA and SDMA interfere with NO synthesis by competing with arginine, the substrate of NOS, for cellular transport across the membrane resulting in an intracellular arginine depletion.\textsuperscript{42} In addition, ADMA and SDMA may compete with arginine for tubular re-absorption in the kidney, thereby reducing arginine availability for NO production.\textsuperscript{43} Whereas SDMA possibly reduces NO syntheses by substrate reduction, ADMA reduces both substrate availability and NOS activity resulting in a larger NO depletion. While the association between ADMA and arterial disease is well established, this association for SDMA is less clear.\textsuperscript{35,38,44} SDMA is mainly cleared by renal filtration and not affected by DDAH (Figure 2).\textsuperscript{32}

In summary, ADMA is an endogenous NOS inhibitor that reduces the availability of the endothelial produced anti-atherosclerotic substance NO. This ADMA-induced reduction in NO is believed to result in unhealthy vessels and an increased arterial disease risk. Oestrogen alone, or combined with a progestogen, influences several important processes associated with arterial disease.\textsuperscript{12-16} Therefore, it is interesting to explore a possible relation between these female hormones and ADMA concentrations in women.

### 3. ADMA, menopause and menopausal therapies

Little is known about the effect of endogenous oestrogens on ADMA concentrations. Twelve weeks after the operation, ADMA concentrations in ovariectomised rats did not differ from those in sham operated rats.\textsuperscript{181} In premenopausal women (mean age 26.8 years (range 21-31 years)) in the low oestrogenic phase (oestradiol less than 100 pg/ml) of their menstrual cycle ADMA concentrations were 48% higher than ADMA concentrations in women (mean age 31.1 years (range 25-36 years)), undergoing ovarian hyperstimulation (oestradiol more than 2000 pg/ml) within an in vitro fertilisation and embryo transfer program.\textsuperscript{182} ADMA concentrations inversely correlated with endogenous oestrogen concentrations in 33 women with coronary heart disease (CHD) (mean age 58 years) and in 17 women without CHD (mean age 54 years).\textsuperscript{183} Postmenopausal women participated in both groups. In a cross-sectional study of 186 women a significantly higher ADMA concentration was observed in women over 50 years of age than in younger women.\textsuperscript{105} Although menopausal status and HT use were not documented in this study, this age-related difference, which was not observed in men, suggests that ADMA levels may increase with the onset of menopause. Up till now, no longitudinal data of ADMA concentrations in women going through the transition of physiological or surgical menopause have been published.

Cell cultures incubated with oestrogens showed lower ADMA concentrations than control cell cultures.\textsuperscript{167,182,184} In male rats, five days of pre-treatment with
17β-oestradiol inhibited a native low-density lipoprotein induced increase in ADMA concentrations. Treatment with oral conjugated equine oestrogens or an ethynyloestradiol implant reduced ADMA concentrations in postmenopausal women. The SERM raloxifene in a dosage of 150 mg per day for two years had no effect. So far, no studies have explored whether or not adding a progestogen modifies the effect of oestrogens on ADMA concentrations. While 17β-oestradiol reduces
4. Objective and Outline of this Thesis

This thesis explores the relation between plasma ADMA concentrations and oestrogens, either endogenous or exogenous, in women. In addition, the effects of several alternatives for HT on ADMA concentrations are investigated as well. The objective of this thesis is:

Do menopause, HT and alternatives for HT modify ADMA concentrations in apparently healthy middle-aged women?

The objective is addressed in the following questions:

- Does menopause, either physiological or surgical, influence ADMA concentrations?
- Does oral 17β-oestradiol therapy influence ADMA concentrations in postmenopausal women?
- Does adding a progestogen change the effect of oral 17β-oestradiol therapy on ADMA concentrations?
- Does the effect of HT on ADMA concentrations depend on the route of administration?
- And finally, are ADMA concentrations influenced by alternatives for HT, such as plant-derived substances or SERMs?

### Effects of life style habits on ADMA blood concentrations

<table>
<thead>
<tr>
<th>life style habit</th>
<th>effect on ADMA</th>
<th>references</th>
</tr>
</thead>
<tbody>
<tr>
<td>smoking*</td>
<td>↑ (129%)</td>
<td>38,98,106,108,109</td>
</tr>
<tr>
<td>alcohol consumption</td>
<td>↑ (≈ 19%)</td>
<td>145</td>
</tr>
<tr>
<td>endurance training</td>
<td>↓ (18%)</td>
<td>146</td>
</tr>
<tr>
<td>high-carbohydrate diet</td>
<td>↓</td>
<td>145,147</td>
</tr>
<tr>
<td>high-fat diet</td>
<td>↑ (≈ 141%)</td>
<td>148</td>
</tr>
<tr>
<td>high-fat diet plus olive oil</td>
<td>no effect</td>
<td>149</td>
</tr>
<tr>
<td>high-fat diet plus walnuts</td>
<td>no effect</td>
<td>149</td>
</tr>
<tr>
<td>high-salt diet</td>
<td>↑ (≈ 15 - 75%)</td>
<td>150,151</td>
</tr>
<tr>
<td>Mediterranean diet</td>
<td>no effect</td>
<td>152</td>
</tr>
<tr>
<td>omega-3 fatty acid supplementation</td>
<td>no effect</td>
<td>152</td>
</tr>
<tr>
<td>weight reduction</td>
<td>↓ (19%)</td>
<td>107</td>
</tr>
</tbody>
</table>

Table 3: * Some studies did not find a difference in ADMA concentrations between smokers and non-smokers and two studies found a reduced ADMA concentration in smokers. ADMA release in cultured cells its effect on plasma ADMA concentrations in postmenopausal women is not yet known. The effects of other non-oral administration routes for oestrogens on ADMA concentrations have not yet been elucidated. Furthermore, the effects of alternatives for HT, like plant extract containing substances with oestrogenic activity or SERMs other than raloxifene, on ADMA concentrations have not yet been published.
<table>
<thead>
<tr>
<th><strong>Effects of therapeutic agents on ADMA blood concentrations</strong></th>
<th><strong>effect on ADMA</strong></th>
<th><strong>references</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ACE-inhibitors and angiotensin receptor blockers</strong></td>
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<td>captopril</td>
<td>↓ (≈ 26 - 48%)</td>
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<tr>
<td>enalapril</td>
<td>↓ (0 - 17%)</td>
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<td>eprosartan</td>
<td>↓ (16%)</td>
<td>154</td>
</tr>
<tr>
<td>losartan</td>
<td>↓ (≈ 14%)</td>
<td>156</td>
</tr>
<tr>
<td>perindopril</td>
<td>↓ (≈ 12 - 16%)</td>
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<tr>
<td>quinapril</td>
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<td>155</td>
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<tr>
<td>valsartan</td>
<td>↓ (38%)</td>
<td>73</td>
</tr>
<tr>
<td>zofenopril</td>
<td>↓ (≈ 24%)</td>
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<tr>
<td><strong>calcium channel blocker</strong></td>
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<tr>
<td>amlodipine</td>
<td>↓ (39%)</td>
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<tr>
<td>nifedipine</td>
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<tr>
<td>nifedipine and cerivastatin</td>
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<tr>
<td><strong>hypoglycaemic agents</strong></td>
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<tr>
<td>metformin</td>
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<tr>
<td>rosiglitazone</td>
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<td>folate</td>
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<td>100</td>
</tr>
<tr>
<td>vitamin E</td>
<td>no effect</td>
<td>177</td>
</tr>
<tr>
<td>vitamin combination preparation</td>
<td>no effect</td>
<td>173</td>
</tr>
<tr>
<td><strong>others</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>iloprost</td>
<td>↓ (≈ 28%)</td>
<td>178</td>
</tr>
<tr>
<td>bisoprolol</td>
<td>no effect</td>
<td>156</td>
</tr>
<tr>
<td>darbepoetin</td>
<td>↑</td>
<td>179</td>
</tr>
<tr>
<td>erythropoietin</td>
<td>↑ (16%)</td>
<td>179</td>
</tr>
<tr>
<td>methylprednisolone</td>
<td>↑ (≈ 18%)</td>
<td>180</td>
</tr>
</tbody>
</table>

Table 4: ACE, angiotensin converting enzyme

\(^a\) Pre-treatment with probucol only prevented an ADMA increase after endothelial injury induced by low-density lipoprotein cholesterol. No effects on ADMA concentrations in control groups.\(^174,176\)
To investigate the influence of physiological menopause on ADMA concentrations we performed a longitudinal study of 16 women going through the physiological menopause and a case-control study in which 27 postmenopausal women were compared with 27 age-matched premenopausal women. The effect of surgical menopause on ADMA concentrations was studied in 11 women who underwent a prophylactic bilateral salpingo-oophorectomy (pBSO). These studies are presented in Chapter 2. Chapter 3 describes the short-term effects of oral 17β-oestradiol therapy either alone or combined with the progestogens dydrogesterone or trimetostone on ADMA concentrations. The difference in effects on ADMA concentrations between transdermally versus orally administered 17β-oestradiol is discussed in Chapter 4 and the comparison between intranasally and orally administered HT in Chapter 5. The effect of gestodene on oral 17β-oestradiol induced changes in ADMA concentrations is also studied in Chapter 6 and 7 describe the effects of a supplement containing phytoestrogens and Actaea racemosa L. on ADMA, lipids and C-reactive protein and the second section examines the effect of this supplement on menopausal symptoms. In Chapter 8 the effect of twelve weeks of HMR 3339, a novel SERM, compared to raloxifene on ADMA concentrations in postmenopausal women is examined. In Chapter 9 the results of the studies in this thesis are discussed and recommendations for future research are postulated.

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1. GENERAL INTRODUCTION AND OUTLINE


1. GENERAL INTRODUCTION AND OUTLINE


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1. GENERAL INTRODUCTION AND OUTLINE


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CHAPTER 2

The influence of physiological and surgical menopause on coronary heart disease risk markers

OBJECTIVE: To investigate the influence of physiological and surgical menopause on serum concentrations of emerging and established coronary heart disease (CHD) risk markers and sex hormones.

METHODS: Changes during the physiological menopausal transition were investigated in two studies. In a longitudinal study, 16 women were followed from two years before until two years after physiological menopause. In a case-control study, 27 early postmenopausal women were compared with 27 age-matched late premenopausal women. Surgical menopause was investigated in 11 women undergoing a prophylactic bilateral salpingo-oophorectomy. The following parameters were measured: serum concentrations of estradiol (E$_2$), follicle-stimulating hormone (FSH), inhibin A, inhibin B, asymmetric dimethylarginine (ADMA), lipids, leptin, homocysteine, C-reactive protein, and coenzyme Q10, as well as weight and body mass index.

RESULTS: Following physiological and surgical menopause, serum E$_2$ and inhibin A and B decreased, whereas FSH increased (all $P < 0.01$). Serum ADMA, total and low-density lipoprotein (LDL-) cholesterol and leptin concentrations were significantly higher in postmenopausal women compared to premenopausal women (all $P < 0.05$). Serum homocysteine concentrations increased significantly during the physiological menopausal transition. Total and LDL-cholesterol increased after the surgical menopause (both $P = 0.01$). None of the other parameters studied was influenced significantly by the menopausal transition. No difference in change in the different CHD risk markers investigated was observed between physiological and surgical menopause.

CONCLUSION: The CHD risk profile was affected unfavorably by both physiological and surgical menopause. Changes in most CHD risk markers were small, despite the substantial changes in hormonal parameters.

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1. Introduction

Natural menopause is marked by a woman’s last vaginal bleeding induced by the influence on the endometrium of hormones produced by the ovaries. After menopause the production of female sex hormones in the ovaries decreases significantly. The decline in endogenous estrogen concentrations can coincide with and is partly responsible for symptoms like hot flushes, night sweats and dryness of the vagina and has been related to long-term effects such as an increased risk for osteoporotic fractures and coronary heart disease (CHD).

The menopausal transition is a physiological process, strongly related to the gradual decline in ovarian function. However, menopause can also be induced by surgical removal of both ovaries. This situation is characterized by an acute decrease in endogenous estrogen serum levels. Women after a surgical menopause have a higher CHD risk than women with a physiological menopause. Therefore, surgically-induced menopause is an interesting model for research on endogenous estrogen-related CHD risk.

Large numbers of women and expensive, complex trials would be needed to study the influence of menopause on hard clinical CHD endpoints. An interesting alternative is to investigate the influence of menopause on CHD risk markers. This can provide insight into the mechanisms through which endogenous estrogens could influence CHD risk and may give direction to prevention or treatment strategies for CHD in women after the menopause. CHD risk markers can be classified according to processes involved in CHD, such as coagulation, lipid metabolism, endothelial function, inflammation, oxidation, and body composition. There is an increasing interest in the CHD risk marker asymmetric dimethylarginine (ADMA), a nitric oxide synthase (NOS) inhibitor. This marker has been particularly studied in predominantly male populations. Coenzyme Q10 (ubiquinone) (Q10) is an interesting antioxidant which could possibly have a positive effect on CHD risk.

As it has been shown that both physiological and surgical menopause increase CHD risk, it was decided to investigate which markers are modified by physiological and surgical menopause. The selection of CHD risk markers was made to enable the investigation of a representative number of processes involved in CHD. Therefore, this study investigated the influence of either type of physiological and surgical menopause on serum concentrations of ADMA, total cholesterol, low-density lipoprotein (LDL-) cholesterol, high-density lipoprotein (HDL-) cholesterol, triglycerides, lipoprotein(a) (Lp(a)), leptin, homocysteine, C-reactive protein (CRP), Q10 as well as on weight and body mass index (BMI). In order to determine hormonal changes around menopause, serum concentrations of estradiol ($E_2$), follicle-stimulating hormone (FSH), inhibin A and B were studied as well.

The influence of physiological menopause was investigated in women with a yearly follow-up from two years before until two years after the menopause and in a case-control study, in which postmenopausal women were compared with age-matched premenopausal women. The influence of surgical menopause was investigated in a group of women with a hereditary high risk of breast and/or ovarian cancer who underwent a prophylactic bilateral salpingo-oophorectomy (pBSO).
2. Materials and methods

2.1. Physiological menopause. The natural menopause was investigated in both a longitudinal study in which 16 women were examined annually from two years before until two years after the menopause, and in a case-control study in which 27 postmenopausal women were compared with 27 age-matched premenopausal women. Menopause was defined as the last menstruation followed by at least 12 months of amenorrhea. The women participating in these two studies were selected from a larger cohort of women who were annually examined in the department of Obstetrics and Gynecology of the VU University Medical Center in Amsterdam, The Netherlands, because of a research program, starting in 1990. Premenopausal women (aged 40 years and over) visiting the outpatient clinic of this institute were invited to participate in this research program to study the menopausal transition. During yearly visits, participants were evaluated for their health and menopausal status, using questionnaires, physical examinations and laboratory measurements. All women gave informed consent before entering the program. Initially, 388 women consented to participate (Figure 1).

Women were excluded for the current studies if they used hormonal contraceptives, postmenopausal hormone therapy (HT) or other therapies for climacteric symptom alleviation or medication interfering with the parameters investigated. Furthermore, women were not allowed to have undergone a unilateral or bilateral oophorectomy or hysterectomy. Among others, the last menstrual date, climacteric symptoms, smoking habits, other health complaints and medication use were documented. Most women (n = 329) were not eligible for the two studies since they started HT during follow-up, underwent a hysterectomy, alone or combined with unilateral or bilateral oophorectomy, or because there was an insufficient number of blood samples available.

For the longitudinal study, women had to have a minimum of three blood samples available with at least one before and one after the menopause. For the
case-control study, blood samples of postmenopausal women were compared with blood samples of age-matched premenopausal women.

2.2. Surgical menopause. Women were selected from a database of women with a hereditary high risk of breast and/or ovarian cancer based on their family history or on an established BRCA1 or BRCA2 germline mutation (Figure 2). These 11 women underwent a pBSO as part of the cancer preventive strategy at the Family Cancer Clinic of the VU University Medical Center, which they visited annually. Informed consent was obtained for blood sampling during these yearly visits for research purposes. For inclusion in this study, the women had to have a regular menstrual cycle at the time of the pBSO and were not allowed to use hormonal contraception, HT or other medication interfering with the parameters investigated. The dates of the pre-pBSO and post-pBSO blood samples were no more than two years from the pBSO date. This study will be further called the pBSO study.

2.3. Risk marker analysis. Venous blood samples were collected between 8:00 a.m. and 10:00 a.m. in the cohort on the physiological menopause and between 8:00 a.m. and 12:00 a.m. in the women with a surgical menopause. In the longitudinal and case-control study women had fasted before blood sampling, whereas the women in the pBSO study had not. Blood was collected in plain tubes. Serum was separated by centrifugation at 1,800 \(g\) at 20°C for 10 minutes within one hour of collection and divided into aliquots and stored at 70°C until analysis.

Serum \(E_2\) concentrations were measured with a radio-immunoassay (Sorin Biomedica, Saluggia, Italy) with a lower detection limit of 18 pmol/L. FSH was determined using a fluorescence immunoassay (Delfia, Wallac Turku, Finland) with a lower detection limit of 0.5 U/L. The intra-assay coefficients of variation (CV) were less than 6% for both measurements. The inter-assay CV was less than 14% for \(E_2\) and less than 6% for FSH measurements.

Inhibin A and B concentrations were measured with a colorimetric immunoassay (Serotec Limited, Oxford, UK). The lower detection limit for inhibin A was
0.1 ng/L and for inhibin B 1.0 ng/L. The inter-assay CVs were less than 11% and 16%, respectively. ADMA concentrations were measured by high-performance liquid chromatography with fluorescence detection. The inter-assay CV was less than 3%.

Serum lipid concentrations were measured with a Modular P. system (Roche, Mannheim, Germany). For total cholesterol, HDL-cholesterol, and triglycerides, the following reagents were used: CHOD-PAP, HDL-C plus, and GPO-PAP, respectively (all by Roche, Mannheim, Germany). The inter-assay CVs were less than 7%. LDL-cholesterol was calculated using the Friedewald formula. Serum Lp(a) concentrations were measured using an enzyme-linked immunoassay (ELISA, Innogenetics NV, Zwijndrecht, Belgium). The intra-assay CV for this ELISA was 3.6% and the inter-assay CV was 4.5%.

Leptin was determined by radio-immunoassay (Linco Research, Inc., St. Charles, USA) with a lower detection limit of 0.5 g/L. The intra- and inter-assay CVs were both less than 9%. Serum total homocysteine concentration was measured by automated fluorescence polarization immunoassay (IMx analyser, Abbott, Illinois, USA), with an inter-assay CV of less than 4%. CRP was assayed by an in-house highly sensitive ELISA with a lower limit of detection of 0.01 mg/L. The intra- and inter-assay CVs were 2.4% and 10.6%, respectively.

The total coenzyme Q10 (sum of oxidized and reduced forms) concentration was determined in serum by liquid chromatography tandem mass spectrometry as described by Hansen et al. Mass spectrometric analysis was performed in the negative ion mode using an API 3000 tandem mass spectrometer (PE Sciex, Toronto, Canada), equipped with an atmospheric pressure chemical ionization interface. Coenzyme Q9 was used as an internal standard at a concentration of 1.0 µmol/L and 1,4-benzoquinone (20 mg/L, final concentration) was used to oxidize the reduced form of Q10 before analysis. The intra- and inter-assay CVs were 2.1% and 3.2%, respectively.

All samples of a given subject in the longitudinal study and the pBSO study and the matched samples in the case-control study were assayed within a single run.

2.4. Statistical analysis. Statistical analysis was performed using the Statistical Package for the Social Sciences PC + 12.0.1 (SPSS Inc., Chicago, Illinois). In all three studies, population characteristics and CHD risk markers are given as mean and standard deviation when normally distributed, or as median (25th - 75th percentile) when the distribution was skewed. In the longitudinal study, analyses of variance (ANOVA) for repeated measurement were used to evaluate changes over time during the four years of follow-up. Paired t-tests were used to evaluate differences between two years before menopause and two years after menopause. When the distribution was skewed the data were log-transformed before using the paired t-test. When the distribution was still skewed after log-transformation, the Wilcoxon signed-rank test was used. Weight was measured in kilograms with one decimal precision. BMI was calculated as weight/(length)^2.

In the longitudinal study, the blood samples were categorized as follows: menopause minus two years (M - 2) = drawn between 1.5 years and 2.5 years before menopause; menopause minus one year (M - 1) = drawn between 6 months and 1.5 years before menopause; menopause (M) = drawn between 6 months before and 6 months after menopause; menopause plus one year (M + 1) = drawn between 6
months and 1.5 years after menopause; menopause plus two years (M + 2) = drawn between 1.5 years and 2.5 years after menopause. Ten women had a complete series of blood samples at all five points in time. The last-observation-carried-forward procedure was used in six cases to supplement the missing data in these women. The group of 10 women was analyzed separately from the group of 16 women. The results of the subgroup of 10 women with complete data are presented, since the results of the group of 16 women did not differ from the results of the group of 10 women.

For the case-control study the paired t-test or chi-squared test was used to evaluate differences between postmenopausal and age-matched premenopausal women. When the distribution was skewed the data were log-transformed. When after log-transformation the distribution was still skewed, the Wilcoxon signed-rank test was used. In the case-control study, the number of smokers in the premenopausal group was significantly lower than in the postmenopausal group. Regression analyses were performed with the group variable and smoking as independent factors to test for the influence of smoking on the parameters studied. For the pBSO study, the Wilcoxon signed-rank test or chi-squared test was used because of the low number of women in this study. To compare the changes in the different CHD risk markers between the case-control and pBSO study, the unpaired t-test or Wilcoxon signed-rank test was used.

In the longitudinal study eight blood samples had undetectable inhibin A concentrations and 31 blood samples had undetectable inhibin B concentrations. In the case-control study, eight women (seven postmenopausal versus one premenopausal) had undetectable inhibin A concentrations and 28 women (21 postmenopausal versus seven premenopausal) had undetectable inhibin B concentrations. In the pBSO study, 11 blood samples (one pre-pBSO samples versus 10 post-pBSO samples) had undetectable inhibin A concentrations and nine blood samples (all post-pBSO samples) had undetectable inhibin B concentrations. In the statistical analyses, samples with a concentration below the detection limit were included, using a concentration of 50% of the detection limit (i.e. 0.05 ng/L for inhibin A and 0.5 ng/L for inhibin B).

Linear mixed model regression analyses were performed between log-transformed serum $E_2$ concentrations and the different CHD risk markers using the pooled results of all three studies.

3. Results

3.1. Longitudinal study. For the longitudinal study, 16 women were eligible (Figure 1). Ten women had complete series of five measurements. The characteristics at menopause of both the 10-women group and the 16-women group are presented in Table 1. These characteristics did not differ between the two groups. At menopause both study populations were on average 51 years of age and had an average BMI of 26 kg/m$^2$ and normal blood pressures. Approximately 40% of the women were cigarette smokers.

The ANOVA over four years of follow-up was significant for a decrease in serum $E_2$ and inhibin A and B concentrations (Table 2 and Figure 3), whereas FSH concentrations increased significantly over these four years (Figure 3). Leptin, weight and BMI did not significantly change over the four years of follow-up (Table 2 and Figure 6). However, the mean leptin concentration of two years after the
3. RESULTS

### Longitudinal study: characteristics of women at the time of menopause

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>n 10*</th>
<th>n 16**</th>
</tr>
</thead>
<tbody>
<tr>
<td>age (years)</td>
<td>51.4 ± 2.4</td>
<td>50.7 ± 2.5</td>
</tr>
<tr>
<td>body mass index (kg/m²)</td>
<td>25.6 ± 2.4</td>
<td>27.0 ± 4.7</td>
</tr>
<tr>
<td>blood pressure systolic (mmHg)</td>
<td>129 ± 17</td>
<td>128 ± 15</td>
</tr>
<tr>
<td>blood pressure diastolic (mmHg)</td>
<td>85 ± 7</td>
<td>84 ± 8</td>
</tr>
<tr>
<td>smoking n (%)</td>
<td>4 (40.0%)</td>
<td>7 (43.7%)</td>
</tr>
</tbody>
</table>

Table 1: Values are given as mean ± standard deviation, or as number of women (%). No significant differences were found between the two groups (P > 0.05).

* n, number of women.
** Women with a complete dataset at the five points in time.

Menopause was significantly higher compared with that of two years before the menopause (12.4 µg/L (9.9 - 20.4 µg/L) versus 11.4 µg/L (6.2 - 14.9 µg/L); P = 0.02).

Homocysteine showed a significant change over time (ANOVA, P = 0.01) with the largest mean increase in the first year after menopause (9.5% (95% confidence interval (CI) 4.5% to 14.5%); P = 0.02) (Figure 7). Serum concentrations of ADMA (Figure 4), the lipids (Figure 5), CRP and Q10 (Figure 7) did not change significantly in this group of women going through the menopausal transition.

#### 3.2. Case-control study

For the case-control study, 27 postmenopausal women were compared with 27 age-matched premenopausal women (Table 3). The postmenopausal women were on average 28 months (12 to 36 months) after the moment of menopause and the premenopausal women were on average 16 months (1 to 54 months) before menopause. The mean age ± standard deviation was 52.6 ± 2.4 years in the postmenopausal group and 52.4 ± 1.3 years in the premenopausal group (P = 0.48). Ten women (37.0%) in the postmenopausal group were cigarette smokers versus three women (11.1%) in the premenopausal group (P = 0.01). Regression analysis revealed no significant influence of smoking on the parameters studied. Therefore, the paired t-test and chi-squared test results are shown in Table 3.

Serum E₂ and inhibin A and B concentrations were significantly lower, whereas FSH concentrations were significantly higher in the postmenopausal group than in the premenopausal group (Table 3). The mean ADMA, total cholesterol and LDL-cholesterol concentrations were higher in the postmenopausal women compared to the premenopausal women (Figure 4 and 5). The mean concentrations of HDL-cholesterol, triglycerides, Lp(a), leptin, homocysteine, CRP, and Q10 as well as weight and BMI were not significantly different between the two groups (Table 3).

#### 3.3. Surgical menopause

Eleven premenopausal women who underwent a pBSO were eligible (Table 4 and 5). The pre-pBSO samples had been taken 3 (1 - 11) months before the pBSO and the post-pBSO samples had been taken 11 (10 - 12) months after the pBSO. At the time of the pBSO, women were on average 44.8 (40.3 - 50.4) years. Four women (36%) in this study were current smokers.
Longitudinal study (n = 10): hormones and CHD risk markers during the menopausal transition

<table>
<thead>
<tr>
<th>M - 2</th>
<th>M - 1</th>
<th>M</th>
<th>M + 1</th>
<th>M + 2</th>
<th>ANOVA*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>hormones:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E₂ (pmol/L)</td>
<td>164 (52 - 245)</td>
<td>181 (22 - 365)</td>
<td>30 (23 - 82)</td>
<td>29 (20 - 35)</td>
<td>25 (21 - 29)††</td>
</tr>
<tr>
<td>FSH (U/L)</td>
<td>27 (9 - 44)</td>
<td>21 (11 - 47)</td>
<td>61 (31.0 - 87)</td>
<td>70 (42 - 86)</td>
<td>65 (42 - 90)†</td>
</tr>
<tr>
<td>inhibin A (ng/L)</td>
<td>3.8 (0.7 - 9.7)</td>
<td>6.4 (0.5 - 18.8)</td>
<td>0.8 (0.4 - 2.4)</td>
<td>0.4 (0.2 - 0.5)</td>
<td>0.3 (0.1 - 0.8)†</td>
</tr>
<tr>
<td>inhibin B (ng/L)</td>
<td>1.5 (0.5 - 16.8)</td>
<td>5.0 (2.5 - 12.3)</td>
<td>1.3 (0.5 - 3.8)</td>
<td>0.5 (0.5 - 1.5)</td>
<td>0.5 (0.5 - 0.6)</td>
</tr>
<tr>
<td><strong>ADMA:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADMA (μmol/L)</td>
<td>0.486 ± 0.078</td>
<td>0.478 ± 0.075</td>
<td>0.485 ± 0.039</td>
<td>0.499 ± 0.059</td>
<td>0.490 ± 0.065</td>
</tr>
<tr>
<td><strong>lipids:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TOT-C (mmol/L)</td>
<td>6.1 ± 1.1</td>
<td>6.0 ± 0.8</td>
<td>6.3 ± 1.0</td>
<td>6.5 ± 1.1</td>
<td>6.4 ± 1.0</td>
</tr>
<tr>
<td>LDL-C (mmol/L)</td>
<td>4.4 ± 1.0</td>
<td>4.2 ± 0.9</td>
<td>4.6 ± 1.0</td>
<td>4.7 ± 1.1</td>
<td>4.6 ± 0.9</td>
</tr>
<tr>
<td>HDL-C (mmol/L)</td>
<td>1.15 ± 0.28</td>
<td>1.16 ± 0.31</td>
<td>1.12 ± 0.35</td>
<td>1.11 ± 0.26</td>
<td>1.21 ± 0.44</td>
</tr>
<tr>
<td>Trigl (mmol/L)</td>
<td>1.1 (0.9 - 1.2)</td>
<td>1.1 (1.0 - 1.4)</td>
<td>1.2 (1.0 - 1.5)</td>
<td>1.2 (0.9 - 1.7)</td>
<td>1.2 (0.9 - 1.8)</td>
</tr>
<tr>
<td>Lp(a) (mg/L)</td>
<td>132 (113 - 265)</td>
<td>141 (118 - 275)</td>
<td>139 (120 - 299)</td>
<td>150 (101 - 354)</td>
<td>157 (121 - 295)</td>
</tr>
<tr>
<td><strong>body composition:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>weight (kg)</td>
<td>68.2 ± 7.1</td>
<td>69.7 ± 9.2</td>
<td>69.2 ± 8.9</td>
<td>70.9 ± 9.8</td>
<td>70.0 ± 10.1</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.4 ± 2.0</td>
<td>25.8 ± 2.8</td>
<td>25.6 ± 2.4</td>
<td>26.2 ± 2.6</td>
<td>26.0 ± 2.7</td>
</tr>
<tr>
<td>leptin (µg/L)</td>
<td>11.4 (6.2 - 14.9)</td>
<td>12.7 (8.2 - 15.4)</td>
<td>12.8 (7.8 - 15.8)</td>
<td>13.3 (8.0 - 15.4)</td>
<td>12.4 (9.9 - 20.4)†</td>
</tr>
<tr>
<td><strong>other CHD risk markers:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hcy (µmol/L)</td>
<td>9.2 ± 1.7</td>
<td>9.4 ± 1.3</td>
<td>9.8 ± 1.8</td>
<td>10.7 ± 2.0</td>
<td>10.0 ± 1.7</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>0.69 (0.46 - 1.31)</td>
<td>0.43 (0.35 - 1.88)</td>
<td>0.76 (0.47 - 1.99)</td>
<td>0.92 (0.38 - 2.37)</td>
<td>1.01 (0.47 - 1.66)</td>
</tr>
<tr>
<td>Q10 (µmol/L)</td>
<td>0.34 ± 0.07</td>
<td>0.33 ± 0.09</td>
<td>0.34 ± 0.07</td>
<td>0.33 ± 0.07</td>
<td>0.39 ± 0.11</td>
</tr>
</tbody>
</table>

Table 2: Values are given as mean ± standard deviation when the parameter was normally distributed or as median (25th - 75th percentile) when skewed distributed.

M ± X: measurements done X years before or after the menopause ± 6 months (see section Materials and Methods).

ADMA, asymmetric dimethylarginine; BMI, body mass index; CHD, coronary heart disease; CRP, C-reactive protein; E₂, estradiol; FSH, follicle-stimulating hormone; Hcy, homocysteine; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; Lp(a), lipoprotein(a); Q10, coenzyme Q10; TOT-C, total cholesterol; Trigl, triglycerides.

*: Analysis of variance for repeated measurements (ANOVA) for changes over the four-year study period.
†: P < 0.05; ††: P ≤ 0.01 paired t-test for difference between M - 2 and M + 2 years.
‡: Two women from the group of 10 women with complete series had insufficient material for the analysis of ADMA concentrations at one time point and one woman for the analysis of HDL-cholesterol and Lp(a). Therefore, ADMA concentration is presented for a group of eight women and the results of HDL-cholesterol and Lp(a) are presented for a group of nine women.
3. RESULTS

Figure 3. (a) $E_2$, (b) FSH, (c) inhibin A and (d) inhibin B concentrations in the longitudinal, the case-control and the pBSO study. #: The value of two years before menopause significantly differed from two years after the menopause (paired t-test; P < 0.05). $E_2$, estradiol; FSH, follicle-stimulating hormone; pBSO, prophylactic bilateral salpingo-oophorectomy; ANOVA, analysis of variance for repeated measurements.
Serum $E_2$ and inhibin A and B concentrations were significantly lower, whereas FSH concentrations were significantly higher after the operation compared with the mean concentrations before the operation (Table 5). Serum total and LDL-cholesterol concentrations were significantly higher after the pBSO (Table 5). The mean concentrations of the other CHD risk markers investigated in this group did not significantly differ between the post-pBSO and pre-pBSO samples.

The serum total and LDL-cholesterol concentrations differed by 23% (95% CI 9% to 38%) and 37% (95% CI 13% to 61%), respectively between the post- and premenopausal women in the case-control study, whereas total and LDL-cholesterol differed 9% (95% CI 2% to 15%) and 17% (95% CI 5% to 28%), respectively between the post-PBSO and pre-pBSO measurements. The percentage differences in total cholesterol and LDL-cholesterol between the case-control study and the pBSO study did not differ significantly from each other. Also the other CHD risk marker changes in the case-control study did not differ significantly from the changes seen in the pBSO study.

3.4. Pooled data. When the data of the three studies were pooled, a negative correlation was observed between ADMA and log-transformed $E_2$ concentrations ($\beta = -0.02; P = 0.01$, Figure 8). Further, negative correlations were found between log-transformed $E_2$ concentrations and total and LDL-cholesterol ($\beta = -0.41; P < 0.001$ and $\beta = -0.42; P = 0.001$, respectively) and between log-transformed $E_2$ concentrations and homocysteine and Lp(a) ($\beta = -0.70$ and $\beta = -0.04$; both $P = 0.01$). None of the other parameters investigated correlated with log-transformed $E_2$ concentrations.

4. Discussion

In this study, after physiological or surgical menopause, serum $E_2$, inhibin A and B concentrations decreased, in conjunction with an increase in FSH concentrations. ADMA, total and LDL-cholesterol, homocysteine and leptin concentrations increased after physiological menopause. After surgical menopause, a significant increase in serum total and LDL-cholesterol concentrations was found. Physiological or surgical menopause was not found to statistically significantly modify any of the other parameters investigated in the present studies.
4. DISCUSSION

Figure 5. (a) total cholesterol, (b) LDL-cholesterol, (c) HDL-cholesterol and (d) triglycerides concentrations in the longitudinal, the case-control and the pBSO study and (e) lipoprotein(a) concentrations in the longitudinal and case-control study. LDL-cholesterol, low-density lipoprotein cholesterol; HDL-cholesterol, high-density lipoprotein cholesterol; Lp(a), lipoprotein(a); pBSO, prophylactic bilateral salpingo-oophorectomy; ANOVA, analysis of variance for repeated measurements.
The changes in the hormonal concentrations found are in line with the expectations for women transforming from premenopausal to postmenopausal. The mean age of the women at the time of physiological menopause was on average 51 years in the longitudinal study, which is in line with earlier observations. The mean age of the women in the pBSO study at the time of surgery was approximately 45 years, which is comparable with the mean age reported for other pBSO populations.

ADMA is an endogenous inhibitor of NOS. An increase in ADMA results in a decreased nitric oxide (NO) production and a decreased NO concentration enhances atherosclerosis. In several patient populations, elevated ADMA concentrations were associated with a high cardiovascular risk. So far, the influence
### Case-control study: characteristics, hormones and CHD risk markers

<table>
<thead>
<tr>
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<th>premenopausal</th>
<th>postmenopausal</th>
<th>P-value*</th>
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<tr>
<td><strong>n</strong></td>
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<td>27</td>
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<tr>
<td><strong>characteristics</strong></td>
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<tr>
<td>age (years)</td>
<td>52.4 ± 1.3</td>
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<td>months from menopause</td>
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<td>RR - systolic (mmHg)</td>
<td>135 ± 15</td>
<td>130 ± 13</td>
<td>0.24</td>
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<tr>
<td>- diastolic (mmHg)</td>
<td>89 ± 11</td>
<td>86 ± 8</td>
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<td>smoking n (%)</td>
<td>3 (11.1%)</td>
<td>10 (37.0%)</td>
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<td><strong>hormones:</strong></td>
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<tr>
<td>E2 (pmol/L)</td>
<td>129 (60 - 518)</td>
<td>25 (22 - 32)</td>
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<tr>
<td>FSH (U/L)</td>
<td>26 (9 - 53)</td>
<td>74 (53 - 87)</td>
<td>&lt; 0.001</td>
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<tr>
<td>inhibin A (ng/L)</td>
<td>7.0 (1.2 - 12.1)</td>
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<td>&lt; 0.001</td>
</tr>
<tr>
<td>inhibin B (ng/L)</td>
<td>9.0 (0.5 - 33.0)</td>
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<td><strong>ADMA:</strong></td>
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</tr>
<tr>
<td>ADMA (µmol/L)</td>
<td>0.447 ± 0.048</td>
<td>0.483 ± 0.056</td>
<td>0.03</td>
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<td><strong>lirips:</strong></td>
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<td>TOT-C (mmol/L)</td>
<td>5.6 ± 1.1</td>
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<td>LDL-C (µmol/L)</td>
<td>3.9 ± 1.0</td>
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<td>HDL-C (µmol/L)</td>
<td>1.04 ± 0.31</td>
<td>1.13 ± 0.34</td>
<td>0.33</td>
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<td>Trigl (mmol/L)</td>
<td>1.1 (0.8 - 1.6)</td>
<td>1.1 (0.8 - 1.5)</td>
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<td>Lp(a) (mg/L)</td>
<td>295 (154 - 515)</td>
<td>254 (172 - 524)</td>
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<td><strong>body composition:</strong></td>
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<td>weight (kg)</td>
<td>70.6 ± 12.3</td>
<td>72.0 ± 13.8</td>
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<td>BMI (kg/m²)</td>
<td>26.4 ± 4.6</td>
<td>26.9 ± 4.5</td>
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<td>leptin (µg/L)</td>
<td>12.4 (8.6 - 16.0)</td>
<td>12.9 (10.9 - 22.3)</td>
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<td><strong>other CHD risk markers:</strong></td>
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<td>Hcy (µmol/L)</td>
<td>9.6 ± 2.0</td>
<td>10.1 ± 1.6</td>
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<tr>
<td>CRP (mg/L)</td>
<td>1.22 (0.44 - 2.68)</td>
<td>1.24 (0.66 - 2.03)</td>
<td>0.65</td>
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<td>Q10 (µmol/L)</td>
<td>0.33 ± 0.12</td>
<td>0.35 ± 0.10</td>
<td>0.39</td>
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</table>

Table 3: Values are given as mean ± standard deviation, as number (n) or as median (25th - 75th percentile). ADMA, asymmetric dimethylarginine; BMI, body mass index; CHD, coronary heart disease; CRP, C-reactive protein; E₂, estradiol; FSH, follicle-stimulating hormone; Hcy, homocysteine; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; Lp(a), lipoprotein(a); n, number of women; Q10, coenzyme Q10; RR, blood pressure; TOT-C, total cholesterol; Trigl, triglycerides.

*: Paired t-test, when skewed distributed log-transformed, when still skewed distributed Wilcoxon signed-rank test was used.

†: ADMA, LDL-C and HDL-C were measured in 26 couples.

of the menopausal transition in general and of endogenous estrogen in particular on ADMA concentrations has not been reported.

ADMA concentrations in ovariectomized rats did not differ from those of sham operated rats twelve weeks after the operation. Plasma ADMA concentrations
inversely correlated with plasma estrogen concentrations in 33 women with CHD (mean age 58 years) and in 17 women without CHD (mean age 54 years). Pre- and postmenopausal women participated in both groups. In a cross-sectional study of 186 women, a significantly higher ADMA concentration was observed in women over 50 years than in younger women. Although neither the menopausal status nor HT use was documented in this study, this age-related difference, which was not observed in men, suggests that ADMA levels increase with the onset of menopause.

This is the first study reporting a difference in ADMA concentrations between post- and premenopausal women. This difference cannot be explained by an age difference. The mean 9.5% increase in ADMA concentrations after menopause is highly similar to the decrease of approximately 8% in ADMA concentrations found after oral postmenopausal estrogen therapy. Furthermore, a negative correlation between ADMA and $E_2$ concentrations was found in the pooled data of the three studies, a finding that strengthens the suggestion that $E_2$ and ADMA concentrations are inversely correlated.

Remarkably, this finding was not confirmed in the longitudinal study. A possible explanation could be that the follow-up time of two years after the menopause is too short. However, the changes in ovarian hormone concentrations in the longitudinal study were already substantial after 2 years. Moreover, after only three months of oral 1 mg 17β-estradiol, a 5% decrease in ADMA concentrations has already been observed.

Another explanation for finding no changes in ADMA concentrations in the longitudinal study could be the small number of women. This would have been relevant when a substantial non-significant change was found. However, ADMA concentrations increased by less than 1% in this study. So, a very large study population would be needed to make this small change statistically significant.

The present study found higher total and LDL-cholesterol concentrations in postmenopausal compared with premenopausal women after both physiological and surgical menopause. Furthermore, negative correlations between serum $E_2$ concentrations and serum total and LDL-cholesterol concentrations were observed in the pooled data of the three studies. The menopausal transition has been associated with changes in the lipid concentrations towards an atherogenic profile. Particularly, total and LDL-cholesterol are higher after menopause. However, decreases in LDL-cholesterol have been described as well, while some studies did not find any influence of menopause on lipids. Whether or not HDL-cholesterol

<table>
<thead>
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<th>pBSO-study: characteristics at the time of operation</th>
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<tr>
<td>n</td>
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<tr>
<td>age at pBSO (years)</td>
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<tr>
<td>smoking n (%)</td>
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<tr>
<td>sample time before pBSO (months)</td>
</tr>
<tr>
<td>sample time after pBSO (months)</td>
</tr>
</tbody>
</table>

Table 4: Values are given as mean ± standard deviation, as number (n) with percentage in parentheses or as median (25th - 75th percentile). pBSO, prophylactic bilateral salpingo-oophorectomy.
and triglycerides change after the menopause is still not clear due to varying reported results of several studies with different methodology.\footnote{32–34,36} We too did not find a consistent change in HDL-cholesterol and triglycerides in the three different studies.

The influence of menopause on leptin was diverse among the different studies in the literature. Postmenopausal women have been reported to have higher leptin concentrations compared to premenopausal women\footnote{38,39} in line with the results of the present longitudinal study. However, other studies have observed no difference between pre- and postmenopausal women,\footnote{34,40} which is in line with the case-control

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure7.png}
\caption{(a) homocysteine and (b) CRP concentrations in the longitudinal, the case-control and the pBSO study and (c) Q10 in the longitudinal and the case-control study. CRP, C-reactive protein; Q10, coenzyme Q10; pBSO, prophylactic bilateral salpingo-oophorectomy; ANOVA, analysis of variance for repeated measurements.}
\end{figure}
and pBSO studies. The reported influence of menopause on weight and BMI varies widely from no influence to increases in both parameters.\textsuperscript{32–36,38–41} The present study did not reveal an influence of the physiological menopause on weight and BMI.

An increase in homocysteine concentrations was found after menopause in the longitudinal study, but this was not confirmed by the case-control and pBSO studies. Furthermore, a negative correlation between \( E_2 \) and homocysteine was found in the pooled data. Others found higher homocysteine concentrations in postmenopausal women compared with premenopausal women.\textsuperscript{42,43} A possible explanation for the different observations between the case-control study and the literature could be the age range. The first study used a 27 to 44 years age range for the premenopausal women and a 50 to 60 years age range for postmenopausal women.\textsuperscript{42} An age-related increase in homocysteine rather than an effect of menopause per se can therefore not be excluded. To correct for the possible age-induced increase in homocysteine, the postmenopausal women in the second study and in our study were age-matched with the premenopausal women. However, the postmenopausal women were on average 5.4 years after the menopause (range 1.3 to 12.8) in the second study,\textsuperscript{43} whereas in our study the women were on average 2.3 years postmenopausal (interquartile range 2 to 3 years). Possibly, in our study the women
Table 5: Values are given as mean ± standard deviation or as median (25th - 75th percentile). ADMA, asymmetric dimethylarginine; CHD, coronary heart disease; CRP, C-reactive protein; E₂, estradiol; FSH, follicle-stimulating hormone; Hcy, homocysteine; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; pBSO, prophylactic bilateral salpingo-oophorectomy; TOT-C, total cholesterol; Trigl, triglycerides; Wilcoxon, Wilcoxon signed-rank test.

had not been postmenopausal for long enough to allow the detection of a difference in homocysteine concentrations compared with the premenopausal women.

Despite oral estrogen therapy has been shown to increase CRP concentrations, physiological or surgical menopause seems not to affect CRP concentrations. The absence of CRP changes in the present study confirms these earlier observations, although a lack of statistical power for detecting menopause-associated changes in CRP cannot be excluded.

An earlier reported higher mean concentration of the antioxidant Q10 in postmenopausal women compared with premenopausal women could not be confirmed in the present study. This might be explained by the different age composition of the population within the two studies. In the study of Palan et al., the postmenopausal women had a mean age of 61.6 ± 10.6 years and the premenopausal women of 38.2 ± 7.9 years. The authors did not correct for age in their analysis. Therefore, an age-related change cannot be excluded. In our study, the postmenopausal women were age-matched with the premenopausal women excluding an age-related change.

Some women in the case-control study had clinically significant abnormal values of total and LDL-cholesterol, triglycerides and BMI. The clinically abnormal values of the different parameters were divided over different participants. In all three studies, women were identified with CRP concentrations above 10 mg/L,
which is considered as the lower threshold level for the existence of an acute inflammation. Re-analysis with exclusion of the women with abnormal values of the above mentioned parameters did not change the results, when compared to the total population.

The changes in the hormones in all three studies and in the literature were consistent and most of the changes in the CHD risk markers were consistent in the three studies and the literature as well. The slightest changes were seen in the longitudinal study. In the first two years of the study, that is the two years directly before the moment of menopause, many women had low premenopausal $E_2$ and high FSH concentrations (Figure 3). Such low $E_2$ concentrations years before the actual moment of menopause have been observed previously. Therefore, possibly not only the follow-up time period after the menopause but also the observation period before the menopause of two years was too short to detect menopause-related changes.

In the pBSO study, normal pre-pBSO $E_2$ concentrations would be expected since the mean age at the time of surgery was 45 years of age. When taken into consideration that the mean age of the physiological menopause is approximately 51 years of age, the pre-PBSO sample was taken approximately six years before the physiological menopause would occur while the premenopausal samples in the physiological menopause studies were taken approximately two years before the menopause. Consequently, a sharper contrast between the pre- and post-values of the parameters investigated in the pBSO study was expected.

After the physiological menopause the ovaries continue to produce androgens. These androgens are aromatized in the fat tissue to estrogens. After surgical menopause the androgen production of the ovaries disappears since the ovaries are removed. Therefore, after a surgical menopause, the source of estrogens from peripheral conversion of androgens is much smaller than after a physiological menopause. Consequently, larger changes in CHD risk markers were expected after surgical menopause than after physiological menopause.

This hypothesis has been confirmed since surgical menopause seems to have a larger influence on lipids than physiological menopause and women have a higher CHD risk after a surgical menopause than women after a physiological menopause. However, the present study found a non-significant larger difference in ADMA, total and LDL-cholesterol concentrations after the physiological menopause than after the surgical menopause. The postmenopausal women in the case-control study were on average 28 months after menopause whereas the post-pBSO samples were taken on average 11 months after the pBSO operation. Therefore, the women had been estrogen-depleted for a longer period after the physiological menopause than the women in the pBSO study, which may explain the larger differences in ADMA, total and LDL-cholesterol concentrations in the former group. Also the mean age difference between the two study populations could play a role in the observed difference between the case-control and pBSO study.

A major strength of the present study is the approach with three different, complementary designs to investigate the influence of menopause on several CHD risk markers. The selection of CHD risk markers was made to enable the study of a representative number of processes involved in CHD. A possible shortcoming is the statistically insufficient number of women in each of the three sub-studies. In particular, conclusions from non-significant changes in the CHD markers investigated
are therefore difficult to draw. On the other hand, significant differences found in each of the small studies point to a real effect that is not age-dependent and might have clinical significance in addition to statistical significance.

In conclusion, after both physiological and surgical menopause, the CHD risk profile tended to change unfavorably even in the first two years following menopause, where particularly ADMA, lipids and homocysteine showed the largest changes. Changes in the majority of the CHD risk markers studied were small, despite the substantial changes present in serum hormone concentrations.

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CHAPTER 3

Effect of hormone replacement therapy on plasma levels of the cardiovascular risk factor asymmetric dimethylarginine: a randomized, placebo-controlled 12-week study in healthy early postmenopausal women

In a prospective, randomized, placebo-controlled 12-week study, we investigated the effect of oral hormone replacement therapy on asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase (NOS), and an independent risk factor for coronary heart disease. The effects on arginine and on symmetric dimethylarginine (SDMA) were also investigated. Sixty healthy early postmenopausal women received daily either placebo (n = 16) or oral 17β-estradiol 2 mg, either unopposed (E₂, n = 16) or sequentially combined with dydrogesterone 10 mg (E₂ + D, n = 14) or trimegestone 0.5 mg (E₂ + T, n = 14). ADMA levels reduced in all active treatment groups. Compared to baseline and placebo, the largest reduction in ADMA levels was observed in the E₂ + T group (-18.7% [95% CI -25.4 to -11.9%] and -21.1% [95% CI -26.2 to -16.1%], at 4 and 12 weeks respectively). At 4 and 12 weeks, in the E₂ + T group arginine levels were significantly reduced as well (-30.9% [95% CI -41.1 to -20.7%] and -36.3% [95% CI -43.1 to -29.5%], respectively), whereas SDMA levels were significantly lower in the E₂ + D group after twelve weeks (-11.6% [95% CI -19.9 to -3.3%]). In conclusion, unopposed oral estradiol and estradiol combined with dydrogesterone or trimegestone reduced plasma levels of the NOS inhibitor ADMA. Whether the reduction of the NOS substrate arginine in the E₂ + T group counteracts the potentially beneficial effect of ADMA reduction or reflects increased NO production remains to be investigated.

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1. Introduction

Since the publication of the results from the ‘Heart and Estrogen/progestin Replacement Study’ (HERS)\(^1,2\) and the ‘Women’s Health Initiative’ (WHI) trial,\(^3\) a net beneficial effect of oral hormone replacement therapy on cardiovascular risk, as suggested by observational studies\(^4\) and studies on cardiovascular risk factors,\(^1,5,6\) seems unlikely. Women with a mean age of 67 and 63 years respectively, were randomized into the HERS and WHI trial to receive either oral combined hormone replacement therapy or placebo. In these studies no cardiovascular benefit\(^2\) and even some evidence of cardiovascular harm\(^3\) was observed.

Although hormone replacement therapy is no longer prescribed for cardiovascular prevention in postmenopausal women, it is still the most effective treatment for women with climacteric complaints. Discussion is ongoing as to whether or not the HERS and WHI results can be extrapolated to younger, early postmenopausal women without signs of arterial disease. Therefore, it is especially important to study the effect of hormone replacement therapy on cardiovascular risk factors in this population.

Nitric oxide (NO) has a vasodilating effect, inhibits platelet aggregation, and suppresses smooth muscle cell proliferation. In postmenopausal women, its levels have been shown to rise during continuously combined oral estrogen plus progestogen replacement therapy,\(^7,8\) during the estrogen-only phase of sequentially combined oral estrogen replacement therapy,\(^9\) but not in the combined phase.\(^9,10\) Possibly the increased endothelium-dependent flow-mediated dilation that has been observed during oral hormone replacement therapy by some investigators,\(^11,12\) but not by others,\(^13,14\) is associated with an increase in NO production or availability. Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of NO synthase (NOS)\(^15\) and a risk factor for acute coronary events in middle-aged men\(^16\) and for overall mortality and cardiovascular events in patients with end-stage renal disease.\(^17\) In addition, in critically ill patients on a surgical intensive care unit, high plasma concentrations of ADMA were associated with an adverse outcome.\(^18\)

Recently, we have found that unopposed conjugated equine estrogens reduce plasma levels of ADMA.\(^19\) Since the effect of estrogen replacement therapy on the cardiovascular system depends on the type of estrogen, the dosage, the route of administration and the addition of a progestogen, we decided to investigate whether unopposed oral 17\(^\beta\)-estradiol also lowers ADMA and whether the addition of the progestogens, dydrogesterone or trimegestone, modulates this effect. In addition, the effects on plasma levels of arginine, a precursor of NO, and symmetric dimethylarginine (SDMA), a stereoisomer of ADMA that does not inhibit NOS, were investigated. Measurements were performed in stored plasma samples of a randomized placebo-controlled 12-week study in healthy early postmenopausal women.\(^20–26\)

2. Subjects and methods

2.1. Subjects. Healthy, nonhysterectomized postmenopausal women were recruited through advertisements in local newspapers; 65 women were enrolled in this 12-week study, which was performed at the outpatient clinic of the Department of
Obstetrics and Gynecology. The investigation conformed to the principles outlined in the Declaration of Helsinki. Written informed consent was obtained from each participant before entering the study. The Institutional Review Board of the VU University Medical Center approved the protocol.

Participants were between 45 and 60 years, smoked fewer than 15 cigarettes per day, were normotensive (less than 160/90 mmHg), had a body mass index (BMI) no greater than 30 kg/m² and had been amenorrhoeic for 6 months to 5 years with serum follicle-stimulating hormone concentrations above 20 IU/L and estradiol concentrations lower than 150 pmol/L. None of the women had received hormone replacement therapy for at least 3 months before entering the study and none took cardiovascular medication. Exclusion criteria included a history of cardiovascular, venous thromboembolic, metabolic, endocrinological, and (pre-) malignant disease, as well as clinically relevant abnormalities in laboratory tests of hematological, renal and hepatic function. Women with fasting serum levels of cholesterol and triglycerides greater than 8 mmol/L and greater than 4 mmol/L, respectively, were also excluded.

2.2. Design. Eligible women were randomly assigned to either placebo (n = 17), or to unopposed micronized 17β-estradiol 2 mg daily (E₂ group; n = 18), or to sequentially combined hormone replacement therapy consisting of micronized 17β-estradiol 2 mg daily plus either dydrogesterone 10 mg daily (E₂ + D group; n = 15; Femoston, Solvay Pharmaceuticals, Weesp, The Netherlands), or trimegestone 0.5 mg daily (E₂ + T group; n = 15; Hoechst Marion Roussel, Romainville-Cedex, France). The progestogens were given for the last 14 days of each 28-day cycle. The pharmacist of the VU University Medical Center (Amsterdam, The Netherlands) manufactured placebo and estradiol as capsules of identical appearance. The tablets of the sequentially combined hormone replacement therapy were put into capsules of identical appearance by Hoechst Marion Roussel (Paris, France). A computerized randomization list was made. Randomization codes were put into sealed envelopes and stored by the pharmacist of the VU University Medical Center. Medication boxes were numbered and allocation was done in sequence. Unblinding was done at the end of the study period. Women assigned to treatment with unopposed estradiol were treated with dydrogesterone 10 mg daily for 14 days to induce a withdrawal bleeding.

Sixty-five participants were initially enrolled. Five women dropped out before the measurement at 4 weeks and were therefore excluded from the analysis (placebo group: n = 1; E₂ group: n = 2; E₂ + D group: n = 1; E₂ + T group: n = 1). Another three women dropped out between 4 and 12 weeks (placebo group: n = 1; E₂ + D group: n = 1; E₂ + T group: n = 1). In these three cases the last-observation-carried-forward procedure was applied for the missing values at 12 weeks. Therefore, the analyses were based on 60 participants. Reasons for drop out have been published previously.

2.3. Blood collection. At baseline and after 4 weeks (cycle 1) and 12 weeks (cycle 3) of follow-up, venous blood samples were taken between 0800h and 1000h. Blood sampling was performed between days 24 and 28 of these cycles, i.e. at the end of the combined estrogen-progestogen phase of the sequential regimens. The subjects had fasted and had refrained from smoking for at least 10 hours and from consuming alcohol for at least 24 hours before sampling. After 20 minutes
of rest, blood was collected into tubes containing EDTA ($K_3$) (Becton Dickinson, Meyren-Cedex, France). The blood samples were immediately placed on ice and centrifuged within one hour of collection at 3000 $g$ and 4°C for 30 minutes. Plasma was snap-frozen and stored at -70°C until analysis.

2.4. Laboratory methods. ADMA, arginine, and SDMA were measured by high-performance liquid chromatography with fluorescence detection. Briefly, 0.2 mL of plasma was mixed with 0.1 mL of a 40-$\mu$mol/L solution of the internal standard monomethylarginine and 0.7 mL PBS. This mixture was applied to Oasis MCX solid-phase extraction columns (Waters, Milford, MA, USA). The columns were consecutively washed with 1.0 mL of 0.1 mol/L HCl and 1.0 mL methanol. Basic amino acids were eluted with 1.0 mL of concentrated ammonia/water/methanol (10:40:50). After evaporation of the solvent under nitrogen, the amino acids were derivatized with ortho-phthaldialdehyde reagent containing 3 mercaptopropionic acid. The derivatives were separated by isocratic reversed-phase chromatography on a Symmetry C18 column (3.9 x 150 mm; 5 $\mu$m particle size; Waters, Milford, MA, USA) at a column temperature of 30°C. Potassium phosphate buffer (50 mmol/L; pH 6.5), containing 8.7% acetonitrile, was used as mobile phase at a flow-rate of 1.1 mL/min. After elution of the last analyte, strongly retained compounds were quickly eluted by a strong solvent flush with acetonitrile. Fluorescence detection was performed at excitation and emission wavelengths of 340 and 455 nm, respectively. All samples from individual patients were analyzed in the same analytical series. The intra-assay coefficients of variation for ADMA, arginine, and SDMA were 1.2%, 0.4%, and 0.8%, respectively. In each participant the plasma arginine/ADMA ratio was determined.

Serum total cholesterol was measured with an auto-analyzer (Boehringer Mannheim, Germany). Serum follicle-stimulating hormone was determined with a specific immunometrical (luminescence) assay (Amerlite, Amersham, Little Chalfont, United Kingdom). Serum total 17β-estradiol was quantified using a double-antibody radioimmunoassay (Sorin Biomedica, Saluggia, Italy) with a lower limit of detection of 18 pmol/L.

The major route of elimination of ADMA is hydrolysis by dimethylarginine dimethylaminohydrolase (DDAH), an enzyme of which the activity may be inhibited by homocysteine. Plasma homocysteine concentrations were determined earlier by high performance liquid chromatography with fluorescence detection according to Fiskerstrand et al.28

2.5. Statistical analysis. Statistical analysis was performed using the Statistical Package for the Social Sciences 9.0 (SPSS Inc., Chicago, Illinois). Baseline characteristics (Table 1) are given as mean ± SD when normally distributed or as median [25th-75th percentile] when the distribution was skewed. Concentrations of the variables investigated are given as mean ± SD. The mean of the individual percentage changes from baseline to 4 and 12 weeks are given as mean and 95% confidence interval (CI). Standard parametric tests were performed (for variables with a skewed distribution after logtransformation). At baseline, BMI differed slightly among the groups (Table 1) and therefore a general linear model for repeated measurements, with the baseline values of the variable under consideration and BMI as constant covariates (ANCOVA), was used for comparisons among and between the
### Descriptive characteristics of the four groups at baseline

<table>
<thead>
<tr>
<th></th>
<th>Placebo group</th>
<th>$E_2$ group</th>
<th>$E_2 + D$ group</th>
<th>$E_2 + T$ group</th>
<th>P-value$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>16</td>
<td>16</td>
<td>14</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>51.4 ± 3.2</td>
<td>53.1 ± 2.8</td>
<td>52.4 ± 3.5</td>
<td>51.7 ± 2.6</td>
<td>0.42</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>25.0 ± 3.1</td>
<td>24.3 ± 2.9</td>
<td>26.3 ± 2.7</td>
<td>26.7 ± 2.7</td>
<td>0.09</td>
</tr>
<tr>
<td>Blood pressure: systolic (mmHg)</td>
<td>121 ± 9</td>
<td>119 ± 15</td>
<td>123 ± 14</td>
<td>124 ± 15</td>
<td>0.82</td>
</tr>
<tr>
<td>Blood pressure: diastolic (mmHg)</td>
<td>70 ± 8</td>
<td>66 ± 8</td>
<td>70 ± 9</td>
<td>70 ± 11</td>
<td>0.48</td>
</tr>
<tr>
<td>Smokers</td>
<td>N (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 (13)</td>
<td>6 (38)</td>
<td>2 (14)</td>
<td>2 (14)</td>
<td>0.24</td>
</tr>
<tr>
<td>Serum cholesterol (mmol/L)</td>
<td>6.2 ± 1.0</td>
<td>6.0 ± 1.1</td>
<td>6.3 ± 0.9</td>
<td>6.3 ± 1.1</td>
<td>0.87</td>
</tr>
<tr>
<td>Serum FSH (U/L)</td>
<td>54.3 ± 22.9</td>
<td>52.8 ± 20.0</td>
<td>48.1 ± 17.0</td>
<td>55.1 ± 11.4</td>
<td>0.88</td>
</tr>
</tbody>
</table>

Table 1: Values are given as number (N) with percentage in parentheses, mean ± SD, or as median [25th-75th percentile].

$^a$ One-way ANOVA or χ²-test for between group differences.

FSH, follicle-stimulating hormone.
3. Results

Table 1 shows demographic characteristics of the four groups at baseline. In each group, except for the $E_2 + T$ group, one woman had an estradiol level above 150 pmol/L. However, as this occurred in combination with a follicle-stimulating hormone level above 35 IU/L, we did not exclude these women from the analysis. Table 2 summarizes the values of the variables investigated during the study period. At baseline there were no significant differences, among the groups, for any of the variables.

ANCOVA over the 12-week study period showed significant changes in ADMA levels in all active treatment groups. The decrease found in the $E_2 + T$ group was significantly larger than the decreases observed in the $E_2$ group and the $E_2 + D$ group (Table 2).

For arginine and the arginine/ADMA ratio, ANCOVA showed significant differences among groups. This could be attributed, both for arginine and the arginine/ADMA ratio, to significant differences between the $E_2 + T$ and the other groups. The decreases in arginine and arginine/ADMA ratio observed after 4 weeks of treatment persisted after 12 weeks.

In addition, ANCOVA showed differences in SDMA among the groups (overall, $P = 0.05$). This was the result of a significant decrease in comparison to placebo in the $E_2 + D$ group and nearly significant decreases in the $E_2$ and $E_2 + T$ groups (Table 2). Figure 1 shows percentage changes from baseline of ADMA, arginine, the arginine/ADMA ratio and SDMA at 12 weeks.

At baseline plasma levels of ADMA showed no significant association with cholesterol ($r = -0.07$; $P = 0.58$). Furthermore, addition of baseline cholesterol as covariate in the ANCOVA analysis did not alter the results shown in Table 2. As described before in more detail, fasting plasma homocysteine concentrations were reduced in all active treatment groups but not in the placebo group. ADMA levels at baseline were not associated with homocysteine. In addition, there were no associations between treatment-induced changes of homocysteine and changes in ADMA levels (data not shown).

4. Discussion

This randomized placebo-controlled study shows that oral 17$\beta$-estradiol sequentially combined with trimegestone reduced levels of ADMA and arginine more than unopposed oral 17$\beta$-estradiol and 17$\beta$-estradiol combined with dydrogesterone. Furthermore, a reduction in arginine/ADMA ratio was found in the $E_2 + T$ group. A significant reduction in SDMA was only observed in the $E_2 + D$ group at 12 weeks.

These results indicate that the effect of unopposed oral 17$\beta$-estradiol 2 mg on ADMA is somewhat smaller than the 8% reduction observed earlier with unopposed oral conjugated estrogens 0.625 mg in a two-year trial. A possible explanation for this may be that the full effect of estrogen was not reached given the relatively short duration of the present study. The finding that the reduction in ADMA was more pronounced at 12 weeks than at 4 weeks in all active treatment groups also points in that direction. Although both progestogens enhanced the effect of 17$\beta$-estradiol,
Levels of ADMA, arginine, SDMA, and the arginine/ADMA ratio during the 12-week study period

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>4 weeks</th>
<th>12 weeks</th>
<th>ANCOVA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ANCOVA&lt;sup&gt;b&lt;/sup&gt;</th>
<th>% Δ 0 - 4&lt;sup&gt;c&lt;/sup&gt;</th>
<th>% Δ 0 - 12&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ADMA (μmol/liter)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>0.46 ± 0.06</td>
<td>0.46 ± 0.07</td>
<td>0.47 ± 0.06</td>
<td>&lt; 0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.45 ± 0.08</td>
<td>0.42 ± 0.06</td>
<td>0.43 ± 0.06</td>
<td>0.03</td>
<td>-3.4 (-11.0 to 4.1)</td>
<td>-5.2 (-11.3 to 0.8)</td>
<td></td>
</tr>
<tr>
<td>E&lt;sub&gt;2&lt;/sub&gt; + D</td>
<td>0.47 ± 0.05</td>
<td>0.44 ± 0.05</td>
<td>0.44 ± 0.03</td>
<td>0.02</td>
<td>-5.1 (-12.3 to 2.0)</td>
<td>-8.0 (-13.4 to -2.6)*</td>
<td></td>
</tr>
<tr>
<td>E&lt;sub&gt;2&lt;/sub&gt; + T</td>
<td>0.46 ± 0.05</td>
<td>0.37 ± 0.05</td>
<td>0.37 ± 0.05</td>
<td>&lt; 0.001</td>
<td>-18.7 (-25.4 to -11.9)**</td>
<td>-21.1 (-26.2 to -16.1)**</td>
<td></td>
</tr>
<tr>
<td><strong>Arginine (μmol/liter)</strong></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>101.4 ± 13.6</td>
<td>99.6 ± 18.9</td>
<td>103.0 ± 13.8</td>
<td>&lt; 0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E&lt;sub&gt;2&lt;/sub&gt;</td>
<td>101.2 ± 12.8</td>
<td>94.9 ± 18.7</td>
<td>95.3 ± 14.3</td>
<td>0.05</td>
<td>-5.3 (-16.6 to 6.0)</td>
<td>-7.3 (-16.0 to 1.4)</td>
<td></td>
</tr>
<tr>
<td>E&lt;sub&gt;2&lt;/sub&gt; + D</td>
<td>94.9 ± 6.7</td>
<td>90.5 ± 10.8</td>
<td>88.8 ± 13.8</td>
<td>0.14</td>
<td>-3.1 (-12.9 to 6.8)</td>
<td>-7.9 (-15.8 to 0.1)</td>
<td></td>
</tr>
<tr>
<td>E&lt;sub&gt;2&lt;/sub&gt; + T</td>
<td>97.4 ± 10.6</td>
<td>65.4 ± 10.6</td>
<td>63.7 ± 10.4</td>
<td>&lt; 0.001</td>
<td>-30.9 (-41.1 to -20.7)**</td>
<td>-36.3 (-43.1 to -29.5)**</td>
<td></td>
</tr>
<tr>
<td><strong>Arginine/ADMA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>222 ± 33</td>
<td>218 ± 35</td>
<td>222 ± 33</td>
<td>&lt; 0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E&lt;sub&gt;2&lt;/sub&gt;</td>
<td>227 ± 32</td>
<td>228 ± 43</td>
<td>227 ± 41</td>
<td>0.75</td>
<td>-1.6 (-9.9 to 6.7)</td>
<td>-1.6 (-12.0 to 8.8)</td>
<td></td>
</tr>
<tr>
<td>E&lt;sub&gt;2&lt;/sub&gt; + D</td>
<td>204 ± 28</td>
<td>207 ± 30</td>
<td>204 ± 38</td>
<td>0.83</td>
<td>3.3 (-6.0 to 12.5)</td>
<td>0.3 (-8.0 to 8.6)</td>
<td></td>
</tr>
<tr>
<td>E&lt;sub&gt;2&lt;/sub&gt; + T</td>
<td>213 ± 25</td>
<td>177 ± 19</td>
<td>171 ± 21</td>
<td>&lt; 0.001</td>
<td>-14.6 (-23.5 to -5.6)*</td>
<td>-19.1 (-25.2 to -13.1)**</td>
<td></td>
</tr>
<tr>
<td><strong>SDMA (μmol/liter)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>0.47 ± 0.07</td>
<td>0.48 ± 0.07</td>
<td>0.48 ± 0.07</td>
<td>0.05</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.49 ± 0.07</td>
<td>0.46 ± 0.08</td>
<td>0.46 ± 0.09</td>
<td>0.05</td>
<td>-7.4 (-15.9 to 1.1)</td>
<td>-7.7 (-16.2 to 0.9)</td>
<td></td>
</tr>
<tr>
<td>E&lt;sub&gt;2&lt;/sub&gt; + D</td>
<td>0.49 ± 0.07</td>
<td>0.46 ± 0.06</td>
<td>0.45 ± 0.07</td>
<td>0.01</td>
<td>-8.1 (-16.3 to 0.1)</td>
<td>-11.6 (-19.9 to -3.3)*</td>
<td></td>
</tr>
<tr>
<td>E&lt;sub&gt;2&lt;/sub&gt; + T</td>
<td>0.47 ± 0.07</td>
<td>0.45 ± 0.08</td>
<td>0.45 ± 0.06</td>
<td>0.05</td>
<td>-7.6 (-15.6 to 0.4)</td>
<td>-7.0 (-15.4 to 1.4)</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Concentrations and ratios are given as mean ± SD at baseline, 4 and 12 wk. P values represent one-way ANOVA, cross-sectional comparison for between-group differences at baseline. * P < 0.01, ** P < 0.001: t-test vs. placebo.
<sup>a</sup> Covariance analysis for repeated measurements for between-group differences with the baseline value of the variable under consideration and BMI as covariate (ANCOVA) over the 12-week study period.
<sup>b</sup> ANCOVA over the 12-week study period: placebo versus treatment. Other significant between-group differences were found for ADMA, arginine, and the arginine/ADMA ratio between the E<sub>2</sub> and E<sub>2</sub> + T group (all P < 0.001), and between the E<sub>2</sub> + D and E<sub>2</sub> + T group (all P < 0.001).
<sup>c</sup>%Δ. Mean and 95% confidence interval of the individual percentage changes compared to baseline and placebo at 4 weeks and at 12 weeks, respectively.
Figure 1. Mean (standard error of the mean) of the individual percentage changes from baseline in ADMA, arginine, and SDMA concentration and of the ratio of plasma arginine to ADMA (arginine/ADMA) in the four study groups at 12 weeks. * P < 0.01, and ** P < 0.001: t-test for between group differences.

This effect was most pronounced for trimegestone. In the population studied here, a difference in effect of dydrogesterone and trimegestone has also been observed with regard to levels of soluble intercellular adhesion molecule-1, soluble vascular cell adhesion molecule-1 and procaryoxypeptidase U (thrombin-activatable fibrinolysis inhibitor). Both progestogens have a high affinity for progestogen receptors; however, dydrogesterone has no estrogenic or androgenic activity, whereas trimegestone has potent antiestrogenic and antiandrogenic activity. This might explain part of the observed differences in their effects on various cardiovascular variables.

ADMA and SDMA are derived from the catabolism of proteins containing methylated arginine residues. When these proteins undergo hydrolysis their methylated arginine residues are released. The observed decrease in ADMA might be the consequence of reduced protein methylation or a diminished catabolism of these proteins. The major pathway for elimination of ADMA is hydrolysis by dimethylarginine dimethylaminohydrolase (DDAH), whereas renal clearance is the main mechanism for elimination of SDMA. Since changes in ADMA appeared to be larger than the changes in SDMA in the E2 + T group, increased renal clearance is not the most likely explanation for the observed decrease in ADMA, whereas changes in DDAH activity seem a more plausible explanation. A decline in DDAH activity appears to be related to oxidative stress. When cultured endothelial cells are exposed to oxidized low density lipoprotein (LDL), ADMA accumulates in the medium at a faster rate than during exposure to a vehicle or native LDL. Estrogens have been
reported to increase the resistance of LDL to oxidation, and therefore, the decrease in ADMA observed in our study could be the result of an increase in DDAH activity. On the other hand, recent data suggest that plasma ADMA levels are not influenced by aggressive lowering of LDL cholesterol by statin treatment, making the link between DDAH activity and LDL less plausible. In the present study baseline plasma cholesterol and ADMA concentrations were not correlated. We did not measure on-treatment cholesterol concentrations and therefore it was not possible to correlate changes of ADMA with changes of cholesterol. However, in a previous study on the long-term effects of estrogen replacement therapy we did not observe such an association. Taken together, it is not likely that the reduction of plasma cholesterol plays a causal role in the ADMA lowering effect of hormone replacement therapy. Another potential mechanism is based on the observation that DDAH activity in endothelial cells is inhibited by homocysteine. This mechanism could potentially provide a link between the homocysteine and ADMA lowering effects of HRT. However, in the present study no associations between baseline values of ADMA and homocysteine were found, and also treatment-induced changes in these variables were not related. It thus seems that this mechanism plays no substantial role in the present study. It should be noted however, that our subjects had fasting homocysteine levels within the normal reference range and therefore we cannot exclude an inhibitory effect of homocysteine on DDAH activity at pathological homocysteine levels.

Strikingly, estradiol combined with trimegestone not only lowered ADMA but also arginine levels, leading to a significantly reduced arginine/ADMA ratio. It is tempting to speculate that arginine levels were reduced by increased consumption of arginine for production of NO as a result of diminished inhibition of NOS by ADMA. There are indications that arginine imported by cells is preferentially used as a substrate for NOS. It has been shown that the arginine transporter and endothelial NOS are co-localized in plasma membrane caveolae, suggesting a direct transfer of extracellular arginine to NOS. Estrogen receptor alpha has also been shown to be partly associated with caveolae in a functional complex with NOS. At the moment it is not clear whether reduced arginine levels cause a reduction of NO production by limiting substrate availability, or are a reflection of increased NO production.

One limitation of the study is the partially blinded design. Investigators knew whether women were randomized to placebo or unopposed estradiol on the one hand or to one of the combined arms on the other hand. However, it is very unlikely that this seriously influenced the results, as none of the investigators nor the laboratory personnel nor the participants were aware of the exact medication.

In conclusion, oral $E_2$, whether unopposed or sequentially combined with dydrogesterone or trimegestone, reduces plasma levels of the cardiovascular risk factor and NOS inhibitor ADMA. This effect was especially pronounced in the $E_2 + T$ group and already apparent after four weeks. Whether the reduction of the NOS substrate arginine in the $E_2 + T$ group counteracts the potentially beneficial effect of ADMA reduction or reflects increased NO production remains to be investigated.

Acknowledgements

The authors thank Ms W.M. van Baal, MD, PhD and Ms H. Kessel, MD, for excellent study coordination and recruitment of participants, Ms. S. de Jong
for performing laboratory analyses, and all women who participated in the study. This work was supported by a research grant from Hoechst Marion Roussel/Wyeth Ayerst International and Grant 97-31 from the Biocare Foundation.

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

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

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

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

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

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1. Introduction

Before the age of fifty, the incidence of coronary heart disease (CHD) is more frequent in men than in women, whereas after menopause the risk for CHD increases exponentially in women.\(^1\,^2\) It has been suggested that endogenous female hormones protect pre-menopausal women from CHD. This has led to the hypothesis that hormone therapy (HT) given to postmenopausal women might protect them from CHD. In observational studies lower rates of CHD were found in women using oestrogens, either unopposed or combined with progestogens, in comparison to women not using HT.\(^3\,^4\) However, two large randomised placebo-controlled trials did not confirm this.\(^5\,^7\) HT was given orally in these clinical trials.\(^5\,^7\) Trials with transdermal 17\(\beta\)-oestradiol, either alone or combined with a progestogen are rare. In a non-blinded, non-placebo controlled, randomised study, a non-significant increase in CHD events was observed in the transdermal hormone-treated group in the first two years.\(^8\)

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

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

Little is known about ADMA plasma concentration in women undergoing the menopausal transition. One study analysed endogenous oestradiol (E\(_2\)) concentrations in 33 women with CHD (mean age 58 years) and in 17 women without CHD (mean age 54 years).\(^21\) A multiple linear stepwise regression analysis showed that ADMA concentrations were inversely related with endogenous E\(_2\) concentrations.\(^21\) Up till now, no longitudinal data of ADMA concentrations in women during the menopausal transition have been published.

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

Therefore, the aim of this study was to compare, in a randomised placebo-controlled study, the effect of transdermal and oral 17\(\beta\)-oestradiol on plasma ADMA concentrations. Since it is known that the addition of progestogens can modulate the effect of estrogens on metabolic and cardiovascular markers,\(^22\,^24\) the effect of the addition of the progestogen gestodene to oral 17\(\beta\)-oestradiol was investigated.
as well. Additionally, this study investigated the effects on plasma concentrations of arginine, a precursor of nitric oxide (NO), and symmetric dimethylarginine (SDMA), a stereoisomer of ADMA that does not inhibit NOS. The ratio between arginine and ADMA is considered important for NOS activity. Supplementation of L-arginine can reduce the inhibiting effect of ADMA on NOS and partly restore NO production.\textsuperscript{25} This is why the arginine/ADMA ratio was calculated as well in this study. Previously, we reported on a variety of CHD risk markers measured in this trial.\textsuperscript{9,10,13−16} We were thus able to study correlations between ADMA and total cholesterol, low-density lipoprotein (LDL)-cholesterol, high-density lipoprotein (HDL)-cholesterol, triglycerides, homocysteine, C-reactive protein (CRP), soluble vascular cell adhesion molecule-1 (sVCAM-1), soluble intercellular adhesion molecule-1 (sICAM-1) and soluble E-selectin (sE-selectin) as well.

2. Materials and methods

2.1. Subjects. The design of this study has been published previously.\textsuperscript{9} Briefly, after screening, 152 eligible healthy postmenopausal women who had undergone a hysterectomy were enrolled in this multi-centre study. The investigation conformed to the principles outlined in the Declaration of Helsinki. All Institutional Review Boards approved the protocol. Written informed consent was obtained from each participant before entering the study.

Participants were between 45 and 65 years old, smoked less than 6 cigarettes per day, had blood pressures below 160/100 mmHg and had a body mass index of 30 kg/m\textsuperscript{2} or less. Postmenopausal status was defined as a serum follicle-stimulating hormone (FSH) concentration above 40 IU/L and an endogenous E\textsubscript{2} concentration lower than 110 pmol/L on each of two different visits in the screening period. Having climacteric symptoms was not an in- or exclusion criterion. Only women requiring hormone treatment because of severe climacteric symptoms were excluded from this study. None of the women had received HT within 6 months before randomisation and none took cardiovascular medication. Exclusion criteria included a history of cardiovascular, thrombo-embolic, metabolic, endocrinological, and malignant disease, as well as clinically relevant abnormalities in laboratory tests.

To maintain blinding of the study medication, a double-dummy approach was used. Eligible women were randomly assigned to either a placebo tablet and placebo patch (placebo group, n = 49), or to transdermal 17β-oestradiol 50 μg daily (Climara; Schering AG, Berlin, Germany) and a placebo tablet (tE\textsubscript{2} group, n = 33), or to oral micronised 17β-oestradiol 1 mg daily and a placebo patch (oE\textsubscript{2} group, n = 37), or to oral micronised 17β-oestradiol 1 mg and gestodene 25 μg daily (one tablet) and a placebo patch (oE\textsubscript{2} + G group, n = 33) given for thirteen 28-day cycles. Medication was manufactured by Schering AG, SBU Fertility Control & Hormone Therapy, Berlin, Germany. We included more women in the placebo group than in the other groups, as we expected more dropouts in this group.

At the visits during the treatment period the women returned the surplus patches and tablets, which were counted for compliance evaluation. Women were considered non-compliant if they returned more then 10% of the number of patches and tablets dispensed. According to this criterion, five women (less than 4%) were non-compliant (placebo, n = 2; tE\textsubscript{2}, n = 2; oE\textsubscript{2} + G, n = 1). Re-analysis excluding these women did not alter the results (data not shown).
2.2. **Blood sampling.** At baseline, in cycle 4 (cycle day 1 to 14) and cycle 13 (cycle day 1 to 28) venous blood samples were taken between 8:00 and 10:00 a.m., with participants in a supine position. The subjects had fasted and refrained from smoking for at least 10 hours and from consuming alcohol for more than 24 hours. After 20 minutes of rest, blood was collected with a Vacutainer system (Becton Dickinson, Meyren, Cedex-France) into cooled tubes containing tri-potassium ethylenediaminetetra-acetic acid ($K_3$ EDTA) (Becton Dickinson, Meylan, Cedex-France). Blood samples were immediately placed on ice and plasma was separated by centrifuging at 3,000 g and 4°C for 30 minutes within one hour of collection. Plasma was divided into aliquots, snap-frozen and stored at -80°C until analysis.

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

The analyses of FSH, endogenous $E_2$, lipids, homocysteine, CRP and adhesion molecules were described previously. The statistical analysis was performed using the Statistical Package for the Social Sciences PC + 10.0.5 (SPSS Inc., Chicago, Illinois). Baseline characteristics are given as mean ± standard deviation when normally distributed or as median (range) when the distribution was skewed. ADMA, arginine and SDMA concentrations and the arginine/ADMA ratio are given as mean ± standard deviation. Standard parametric tests were performed. Analyses of covariance (ANCOVA) for repeated measurements, with the baseline value of the variable under consideration, endogenous $E_2$ concentration and smoking as constant covariates, were used for comparisons among and between the groups. The means of the individual percentage changes from baseline are given with 95% confidence interval (CI). A two-tailed $P < 0.05$ was accepted as the level of significance.

No blood samples were available of seven women (placebo, $n = 1$; $tE_2$, $n = 1$; $oE_2$, $n = 3$ and $oE_2 + G$, $n = 2$). Furthermore, three women of whom only baseline samples were available (placebo, $n = 2$ and $tE_2$, $n = 1$) and one woman, who had no data at baseline (placebo, $n = 1$), were excluded from the analyses. For the missing results in another nine women in cycle 13, the last-observation-carried-forward procedure was applied using the results obtained at cycle 4 (placebo, $n = 3$; $tE_2$, $n = 2$; $oE_2$, $n = 2$ and $oE_2 + G$, $n = 2$). Therefore, the analyses of ADMA and arginine were based on 141 women (placebo, $n = 45$; $tE_2$, $n = 31$; $oE_2$, $n = 34$; and $oE_2 + G$, $n = 31$). In view of the fact of a lack of material, the results for SDMA were not obtainable in several samples and as a result the analyses of SDMA were based on 137 women (placebo, $n = 44$; $tE_2$, $n = 29$; $oE_2$, $n = 33$; and $oE_2 + G$, $n = 31$ women). Associations of age, BMI, blood pressure, FSH, and endogenous $E_2$ with baseline concentrations of ADMA were assessed by calculating Pearson’s correlation coefficient. Furthermore, correlations were calculated between baseline values of ADMA and baseline values of arginine, SDMA, and several other relevant factors, measured previously in this study (total cholesterol, LDL- and HDL-cholesterol, triglycerides, homocysteine, CRP, sVCAM-1, sICAM-1 and sE-selectin). Correlations between the absolute
3. Results

The trial profile, with the number of women in each treatment group at baseline and at cycles 4 and 13, is shown in Figure 1. Demographic characteristics of the sub-population analysed (n = 141) did not differ significantly from the original population of 152 women. At baseline, no significant differences were found between the groups in either demographic characteristics or in any of the variables investigated (Table 1). Although all women had endogenous $E_2$ concentrations below 110 pmol/L twice during screening, thirteen women had endogenous $E_2$ concentrations above 150 pmol/L at baseline. These women were distributed as follows: four women in the placebo group (175, 246, 396 and 402 pmol/L); four women in the $tE_2$ group (170, 207, 264 and 912 pmol/L); one woman in the $oE_2$ group (192 pmol/L);
4. EFFECT OF ORAL VERSUS TRANSDERMAL HT ON ADMA

Characteristics of the four groups at baseline

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

Table 1: Values are given as number (N) with percentage in parentheses, as mean ± standard deviation, or as median (range). There were no statistically significant differences between the four groups in the baseline characteristics. \( tE₂ = \text{transdermal 17}\beta\text{-oestradiol 50} \mu\text{g; } oE₂ = \text{oral 17}\beta\text{-oestradiol 1 mg; } oE₂ + G = oE₂ + \text{gestodene 25} \mu\text{g; FSH = follicle-stimulating hormone; } E₂ = 17\beta\text{oestradiol.} \)

and four women in the \( oE₂ + G \) group (153, 169, 173 and 333 pmol/L). Re-analysis without these women did not change the results (data not shown). Therefore, these women were included in the analysis. To correct for potential confounding by differences in baseline endogenous \( E₂ \) concentrations and smoking habits (placebo, \( n = 7; \) \( tE₂, n = 1; \) \( oE₂, n = 3; \) \( oE₂ + G, n = 4; \) \( P = 0.36, \) Fisher’s exact test), we added these two factors as well as the baseline value of the variables investigated into the ANCOVA as constant covariate. Neither (serious) adverse events nor reasons for drop-out were associated with venous thrombo-embolic disease. Three women reported an episode of chest pain (\( tE₂ \) group, \( n = 1; \) \( oE₂, n = 3; \) \( oE₂ + G, n = 4; \) \( P = 0.036, \) Fisher’s exact test), whereas in the other two women this could not be excluded with certainty.

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

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

At baseline, ADMA concentrations were only significantly associated with arginine concentrations (\( r = 0.30; \) \( p < 0.001 \)). The absolute changes in ADMA at cycle 13 correlated with the changes in arginine and SDMA (both \( r = 0.36; \) \( P = 0.001 \)). The baseline concentrations of ADMA did not correlate with the baseline characteristics or the baseline values of any of the CHD markers that were measured previously in this study, i.e. lipids, homocysteine, CRP and adhesion
4. Discussion

This randomised, placebo-controlled trial showed that transdermal as well as oral 17β-oestradiol therapy effectively reduced plasma ADMA concentrations after 13 cycles of treatment. Oral administration of 17β-oestradiol induced a larger decrease in ADMA concentrations than transdermal administration. Gestodene did not reduce ADMA concentrations in either oral 17β-oestradiol therapy group. In addition, the absolute change in ADMA concentrations at cycle 13 was not correlated with any of the absolute changes of these CHD markers.

Table 2: Concentrations and ratios are given as mean ± standard deviation.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Baseline</th>
<th>Cycle 4</th>
<th>Cycle 13</th>
<th>ANCOVAa</th>
<th>ANCOVAb</th>
<th>% Δ 0 - 4a</th>
<th>% Δ 0 - 13b</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADMA (μmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>0.45 ± 0.04</td>
<td>0.46 ± 0.05</td>
<td>0.46 ± 0.05</td>
<td>0.045</td>
<td>-0.8 (-4.3 to 2.6)</td>
<td>-4.0 (-7.5 to -0.6)</td>
<td></td>
</tr>
<tr>
<td>tE2</td>
<td>0.45 ± 0.04</td>
<td>0.45 ± 0.04</td>
<td>0.43 ± 0.03</td>
<td>0.05</td>
<td>&lt; 0.001</td>
<td>-6.3 (-9.9 to -2.7)</td>
<td>-7.7 (-10.9 to -4.4)</td>
</tr>
<tr>
<td>oE2</td>
<td>0.44 ± 0.04</td>
<td>0.42 ± 0.04</td>
<td>0.41 ± 0.04</td>
<td>0.001</td>
<td>-8.1 (-11.6 to -4.5)</td>
<td>-7.5 (-10.8 to -4.3)</td>
<td></td>
</tr>
<tr>
<td>oE2 + G</td>
<td>0.47 ± 0.05</td>
<td>0.43 ± 0.05</td>
<td>0.43 ± 0.05</td>
<td>&lt; 0.001</td>
<td>-6.3 (-9.9 to -2.7)</td>
<td>-7.7 (-10.9 to -4.4)</td>
<td></td>
</tr>
<tr>
<td>Arginine (μmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>99.9 ± 17.4</td>
<td>98.6 ± 17.7</td>
<td>103.6 ± 18.8</td>
<td>0.07</td>
<td>2.6 (-2.0 to 7.2)</td>
<td>-2.4 (-7.9 to 3.0)</td>
<td></td>
</tr>
<tr>
<td>tE2</td>
<td>96.2 ± 12.1</td>
<td>97.4 ± 12.2</td>
<td>95.6 ± 11.4</td>
<td>0.03</td>
<td>-1.2 (-6.3 to 3.9)</td>
<td>-7.9 (-12.5 to -3.3)</td>
<td></td>
</tr>
<tr>
<td>oE2</td>
<td>98.9 ± 13.7</td>
<td>96.0 ± 13.0</td>
<td>94.4 ± 14.0</td>
<td>0.001</td>
<td>-7.9 (-12.1 to -3.7)</td>
<td>-10.6 (-15.3 to -5.9)</td>
<td></td>
</tr>
<tr>
<td>oE2 + G</td>
<td>97.0 ± 12.4</td>
<td>88.2 ± 11.5</td>
<td>89.6 ± 13.3</td>
<td>0.04</td>
<td>-2.9 (-7.7 to 1.8)</td>
<td>-5.1 (-10.2 to 0.0)</td>
<td></td>
</tr>
<tr>
<td>SDMA (μmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>0.47 ± 0.07</td>
<td>0.47 ± 0.08</td>
<td>0.48 ± 0.07</td>
<td>0.06</td>
<td>-1.3 (-6.4 to 3.9)</td>
<td>-5.2 (-11.1 to 0.7)</td>
<td></td>
</tr>
<tr>
<td>tE2</td>
<td>0.45 ± 0.05</td>
<td>0.44 ± 0.05</td>
<td>0.44 ± 0.06</td>
<td>0.09</td>
<td>-4.2 (-9.2 to 0.9)</td>
<td>-6.4 (-12.0 to -0.8)</td>
<td></td>
</tr>
<tr>
<td>oE2</td>
<td>0.46 ± 0.07</td>
<td>0.44 ± 0.08</td>
<td>0.44 ± 0.07</td>
<td>0.01</td>
<td>-2.9 (-7.7 to 1.8)</td>
<td>-5.1 (-10.2 to 0.0)</td>
<td></td>
</tr>
<tr>
<td>oE2 + G</td>
<td>0.47 ± 0.05</td>
<td>0.45 ± 0.07</td>
<td>0.46 ± 0.07</td>
<td>0.10</td>
<td>-0.1 (-5.1 to 4.2)</td>
<td>-3.3 (-8.2 to 1.7)</td>
<td></td>
</tr>
<tr>
<td>Arginine/ADMA ratio</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>220 ± 34</td>
<td>216 ± 31</td>
<td>224 ± 35</td>
<td>0.06</td>
<td>3.3 (-2.0 to 8.6)</td>
<td>1.7 (-3.7 to 7.1)</td>
<td></td>
</tr>
<tr>
<td>tE2</td>
<td>216 ± 26</td>
<td>219 ± 23</td>
<td>223 ± 27</td>
<td>0.41</td>
<td>5.2 (-0.4 to 10.8)</td>
<td>-0.1 (-5.1 to 4.2)</td>
<td></td>
</tr>
<tr>
<td>oE2</td>
<td>224 ± 35</td>
<td>232 ± 38</td>
<td>229 ± 33</td>
<td>0.09</td>
<td>0.1 (-4.9 to 5.2)</td>
<td>-3.3 (-8.2 to 1.7)</td>
<td></td>
</tr>
<tr>
<td>oE2 + G</td>
<td>211 ± 34</td>
<td>208 ± 35</td>
<td>208 ± 33</td>
<td>0.31</td>
<td>0.19</td>
<td>0.42</td>
<td></td>
</tr>
</tbody>
</table>

% Δ percentage changes and are given as mean ± standard deviation.

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Fig. 2. Mean of the individual percentage changes from baseline in plasma ADMA, arginine, and SDMA concentration in the four groups at cycle 13. ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine; tE2, transdermal 17β-oestradiol 50 µg; oE2, oral 17β-oestradiol 1 mg; oE2 + G, oE2 plus gestodene 25 µg. *P < 0.05 and **P < 0.001: t-test for between group differences.

added continuously to oral 17β-oestradiol did not modify the oE2-induced change in ADMA concentrations. Compared to placebo, both oral treatment regimens reduced arginine concentrations significantly, whereas transdermal 17β-oestradiol did not. While the reduction of SDMA in the three treatment groups was comparable, only the reduction in the oE2 group reached statistical significance at cycle 13. The average reduction versus placebo in ADMA induced by oral 17β-oestradiol 1 mg, either given unopposed or continuously combined with gestodene, observed in this study (≈ 7%) is similar to the reduction found earlier after treatment with oral 17β-oestradiol 2 mg (≈ 5%) and oral CEE 0.625 mg treatment (≈ 7%) published previously by our group.22,23 In these two previous studies and the present study, ADMA concentrations were significantly reduced by oral estrogens to a similar extent, indicating that the moderate ADMA lowering effect of estrogens is not a spurious finding but a real phenomenon. Although the absolute effect may seem small, it is noteworthy that the biological variation of plasma ADMA concentrations is also very low, with an inter-individual coefficient of variation of approximately 10%. In the study by Valkonen et al.18 it was shown that even slightly increased ADMA concentrations (above 0.62 µmol/L) were associated with a strongly elevated risk for acute coronary events. Therefore, in our opinion the moderate reductions in
ADMA levels as observed in the present and previous studies may be of clinical relevance.

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

Both ADMA and SDMA are formed by methylation of arginine residues in proteins by a family of protein arginine methyltransferases (PRMTs). Free ADMA and SDMA, as measured in this study, are released upon proteolysis of these methylated proteins. SDMA is mainly cleared by renal excretion, whereas only a small part of ADMA is cleared from the circulation by this pathway. An increase in glomerular filtration rate as a cause for the ADMA-lowering effect of HT can thus be excluded, because this would affect SDMA concentrations to a larger extent than ADMA concentrations. Approximately 80% of ADMA is metabolized by the widely expressed enzyme dimethylarginine dimethylaminohydrolase (DDAH). DDAH is very sensitive to oxidative stress since the active site of the enzyme contains a critical sulfhydryl group that is required for its catalytic activity. Consequently, pathologic stimuli that induce oxidative stress, such as hyperhomocysteinemia and oxidized LDL, may reduce DDAH activity and lead to an accumulation of ADMA. Conversely, compounds with anti-oxidant properties, like oestrogens, may protect DDAH from inactivation by oxidants, leading to a reduction of ADMA concentrations. Several in vitro and in vivo studies on the effect of oestrogens on ADMA metabolism have been conducted. Treatment of rats with 17β-oestradiol was shown to attenuate the increase in plasma ADMA concentrations after injection of LDL. Exposure of human and murine endothelial cells to 17β-oestradiol resulted in an increase in the activity of DDAH and was accompanied by a reduced release of ADMA. All in all, it seems plausible that HT causes an increase of DDAH activity, either directly or indirectly, i.e. by lowering LDL-cholesterol and/or homocysteine concentrations. Although in the present study LDL-cholesterol was significantly reduced after 13 cycles of treatment, absolute reductions of ADMA and LDL-cholesterol were not correlated.

No correlations were found for baseline concentrations of ADMA and homocysteine or the absolute changes after 13 cycles of both variables. It is possible that in comparison to homocysteine other parameters are more dominant in affecting ADMA levels. Results obtained in studies looking at the course of ADMA concentrations after oral methionine loading are conflicting. Although an increased activity of DDAH is consistent with the results of the present study and earlier published data, it affords no explanation for the slight decrease of SDMA concentrations. Although only significant in the oE2 group, the decrease in the other active treatment groups was of a similar magnitude. A similar non-significant decrease of SDMA concentrations was previously observed by us and by Holden et al., suggesting that the reduction of SDMA by HT is a real effect. Oxidative stress may lead to accelerated proteasomal degradation of proteins. It is thus conceivable that reduction of oxidative stress by HT, by reducing proteolysis of methylated proteins,
leads to a diminished generation of both ADMA and SDMA. The combination of a decreased proteolysis and enhanced DDAH activity would explain the decrease of both ADMA and SDMA, the reduction of ADMA being more pronounced.

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

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

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

Acknowledgements

The authors wish to thank Mrs. M.S. Post, MD and Mrs. H. Kessel, MD, for excellent logistical assistance and recruitment of participants (VU University Medical Center, Amsterdam), and the following investigators who participated in this study: J.M.W.M. Merkus, MD, PhD, C.P.T. Schijf, MD, Mrs. C.F. van Het-eren, MD, J.M. Smeenk, MD (University Medical Centre Sint Radboud, Nijmegen); M.V.A.M. Kroeks, MD, PhD (Diakonessenhuis, Utrecht); H.R. Franke, MD, PhD (Medisch Spectrum Twente Hospital Group, Enschede); Mrs. S. de Jong (VU University Medical Center, Amsterdam) for performing laboratory analyses, and all women who participated in the study.

References


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CHAPTER 5

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

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/NET A 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 to 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.

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

Atherosclerosis, in press
1. 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 ($E_2$) (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 $E_2$-exposure was observed. As successor of the $E_2$-only spray, a new intranasal spray for continuous combined 17β-estradiol and norethisterone ($E_2$/NET) administration has been developed. As hepatic metabolism is largely bypassed, it is plausible that, just as with transdermal patches, the intranasal $E_2$/NET spray would have limited effect on ADMA concentrations. The assumption that intranasal $E_2$ 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 $E_2$/NET would be less than after oral $E_2$/NETA, since it is assumed that intranasal $E_2$/NET has less effect on the liver. Therefore, we performed this study comparing the effect of intranasal $E_2$/NET formulation with oral low-dose continuous combined $E_2$/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 sub-study (n = 90) among participants in two Dutch centers. This is the latest sub-study in a series of publications generated from this large international study.
2. Materials and methods

2.1. Participants. This sub-study 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 sub-study.

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 lower than 110 pmol/L and follicle-stimulating hormone (FSH) above 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 or less and of triglycerides of 3.0 mmol/L or less.

Exclusion criteria were a body mass index (BMI) above 32 kg/m$^2$, 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.

2.2. Study design. After a one to six 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 (E$_2$/NET) (S21405, Servier, Courbevoie, France) and one placebo capsule (intranasal group) or one capsule containing 1 mg 17β-estradiol combined with 0.5 mg norethisterone acetate (E$_2$/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 E$_2$ was similar to that of 2 mg oral E$_2$. 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. Comparing intranasal E$_2$ alone with intranasal E$_2$ combined with NET showed a decrease of the bioavailability of estradiol. It was shown that the exogenous estradiol exposure of 345 µg E$_2$ combined with NET was equivalent to the exogenous estradiol exposure after 300 µg E$_2$ alone. In this study, in line with the current recommendation to
use low-dose HT, half the dosage of 345 µg intranasal \( E_2 \) was administered, corresponding with 1 mg oral \( E_2 \) 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.

2.3. 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 (\( K_3 \) EDTA) (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 2,000 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.

2.4. 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 (25\(^{th} \) to 75\(^{th} \) 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 \( \chi^2 \)-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 samples 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 \( E_2 \) with baseline concentrations of ADMA by calculating Pearson’s correlation
3. 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 sub-study. Ninety women (intranasal: n = 47; oral: n = 43) were found eligible for the current sub-study (Fig. 1). At baseline, no significant differences were found between the groups in either demographic characteristics or in any of the variables investigated (Table 1). 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) (Fig. 1). Premature study discontinuation was mainly related to the occurrence of an adverse event.16 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 (DVT). One

<table>
<thead>
<tr>
<th>Characteristics of the two groups at baseline</th>
<th>intranasal</th>
<th>oral</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>47</td>
<td>43</td>
</tr>
<tr>
<td>age (years)</td>
<td>57.5 ± 5.2</td>
<td>55.9 ± 3.9</td>
</tr>
<tr>
<td>amenorrhea (months)</td>
<td>74 (51 to 123)</td>
<td>73 (45 to 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>
</tr>
<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>smokers: 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 FSH (IU/L)</td>
<td>83 ± 32</td>
<td>82 ± 25</td>
</tr>
<tr>
<td>serum endogenous estradiol (pmol/L)</td>
<td>62 (43 to 83)</td>
<td>56 (44 to 91)</td>
</tr>
</tbody>
</table>

Table 1: Values are given as mean ± standard deviation, as median (25th to 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.
Figure 1. Clinical trial profile. The numbers of women in the sub-study 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.

DVT was confirmed (intranasal group) while the other could not be confirmed by 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 2 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; Fig. 2).

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 2). The ANOVA for within-group changes in SDMA in
### Concentrations of ADMA, arginine, SDMA, and the arginine/ADMA ratio during 52 weeks of treatment

<table>
<thead>
<tr>
<th></th>
<th>baseline</th>
<th>week 12</th>
<th>week 52</th>
<th>ANCOVA†</th>
<th>ANOVA‡</th>
<th>% change 0 - 12§</th>
<th>% change 0 - 52¶</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ADMA (µmol/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>intranasal</td>
<td>0.446 ± 0.049</td>
<td>0.438 ± 0.042</td>
<td>0.439 ± 0.042</td>
<td>0.45</td>
<td>-1.3</td>
<td>(-4.3 to 1.8)</td>
<td>-0.8 (-3.7 to 2.1)</td>
</tr>
<tr>
<td>oral</td>
<td>0.462 ± 0.070</td>
<td>0.430 ± 0.063**</td>
<td>0.431 ± 0.063**</td>
<td>&lt; 0.001</td>
<td>-6.3</td>
<td>(-9.3 to 3.2)</td>
<td>-7.4 (-10.4 to -4.4)</td>
</tr>
<tr>
<td>P-value**</td>
<td>0.23</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>arginine (µmol/l)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>intranasal</td>
<td>110.7 ± 19.1</td>
<td>103.3 ± 16.2**</td>
<td>107.9 ± 18.7</td>
<td>&lt; 0.01</td>
<td>-6.1</td>
<td>(-9.1 to -3.0)</td>
<td>-1.6 (-7.6 to 4.3)</td>
</tr>
<tr>
<td>oral</td>
<td>107.0 ± 17.5</td>
<td>99.3 ± 16.8*</td>
<td>105.3 ± 16.0</td>
<td>0.03</td>
<td>-6.5</td>
<td>(-10.9 to -2.1)</td>
<td>-1.7 (-6.4 to 3.0)</td>
</tr>
<tr>
<td>P-value**</td>
<td>0.36</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SDMA (µmol/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>intranasal</td>
<td>0.501 ± 0.066</td>
<td>0.487 ± 0.062</td>
<td>0.493 ± 0.065</td>
<td>0.23</td>
<td>-2.2</td>
<td>(-5.3 to 1.0)</td>
<td>-0.9 (-4.2 to 2.3)</td>
</tr>
<tr>
<td>oral</td>
<td>0.493 ± 0.076</td>
<td>0.472 ± 0.064*</td>
<td>0.478 ± 0.063*</td>
<td>0.03</td>
<td>-3.4</td>
<td>(-6.9 to 0.0)</td>
<td>-4.0 (-8.5 to 0.5)</td>
</tr>
<tr>
<td>P-value**</td>
<td>0.61</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>arginine/ADMA ratio</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>intranasal</td>
<td>246 (221 to 276)</td>
<td>232 (212 to 265)*</td>
<td>236 (220 to 264)</td>
<td>0.13</td>
<td>-6.4</td>
<td>(-10.0 to -2.5)</td>
<td>-4.8 (-10.1 to 1.5)</td>
</tr>
<tr>
<td>oral</td>
<td>226 (207 to 260)</td>
<td>229 (209 to 246)</td>
<td>247 (215 to 268)*</td>
<td>0.03</td>
<td>-2.4</td>
<td>(-6.8 to 2.7)</td>
<td>4.5 (-0.0 to 9.6)</td>
</tr>
<tr>
<td>P-value**</td>
<td>0.10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Concentrations are given as mean ± standard deviation and ratios are given as median (25th to 75th percentile). ADMA = asymmetric dimethylarginine; SDMA = symmetric dimethylarginine; 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.

†: 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).
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 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-, HDL<sub>2</sub>- and HDL<sub>3</sub>-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.

4. 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. 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%. 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. 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. 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). 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. This may provide an explanation for the smaller reduction after transdermal HT administration (4%) than after oral administration (approximately 8%) described earlier. 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. 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.\textsuperscript{27}

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 $\mu$g intranasal $E_2$ was similar to that of 50 $\mu$g transdermal and 2 mg oral.\textsuperscript{19} The dosage of the transdermal therapy used in the previous study, which found a 4\% reduction in ADMA concentrations by transdermal $E_2$, was 50 $\mu$g daily.\textsuperscript{12} In this study, in line with the current recommendation to use low-dose HT, half the dosage of $E_2$ was administered intranasally and this would correspond with 25 $\mu$g $E_2$ 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\textsuperscript{12} 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.\textsuperscript{28} The average reduction in ADMA induced by oral $E_2$/NETA in this study (approximately 7\%) is similar to the reductions after $E_2$ alone or $E_2$ combined with gestodene and dydrogesterone.\textsuperscript{11,12} Gestodene and dydrogesterone did not modify the effect of oral $E_2$ on ADMA concentrations.\textsuperscript{11,12} From these earlier observations and the results of the present study it is plausible that NET does not modify the oral $E_2$ induced ADMA reductions and that it is unlikely that NET would have modulated the effect of intranasal $E_2$.

The studies describing high ADMA concentrations in patients with CHD or high CHD risk included mostly men.\textsuperscript{5–8} 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).\textsuperscript{9} 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.\textsuperscript{9} Schulze et al. observed a significantly higher ADMA concentration in women over 50 years than in younger women.\textsuperscript{29} 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\textsuperscript{30,31} 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.\textsuperscript{32} 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.\textsuperscript{33}

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.\textsuperscript{10–12} The sample size calculation was based on changes in normalized APC sensitivity ratio.\textsuperscript{16} 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 $E_2/NETA$, and not by intranasal $E_2/NET$ 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.

Acknowledgements

The authors wish to thank Mrs. T.E. 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. D.D.M. 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.

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References


5. EFFECTS OF INTRANASAL VERSUS ORAL HT ON ADMA


(20) S 21405 Investigator’s Brochure. version no 3 of 22.02.01. 22-2-2001 2001; Institut de Recherches Internationales Servier, Courbevoie, France.


CHAPTER 6

Effects of a supplement containing isoflavones and *Actaea racemosa* L. on asymmetric dimethylarginine, lipids and C-reactive protein in menopausal women

**Objective:** To investigate the effects of a supplement containing soy isoflavones and *Actaea racemosa* L. on several coronary heart disease (CHD) risk markers in menopausal women.

**Design:** Randomized, placebo-controlled, double-blind study.

**Setting:** Nine hospitals in The Netherlands.

**Patient(s):** One hundred twenty-four menopausal women.

**Intervention(s):** Daily placebo (*n* = 64) or supplement containing soy isoflavones and *Actaea racemosa* L. (*n* = 60) for 12 weeks.

**Main Outcome Measures(s):** Fasting blood concentrations of asymmetric dimethylarginine (ADMA), lipids and C-reactive protein (CRP) at baseline and week 12.

**Results:** In the supplement group, total cholesterol and low-density lipoprotein cholesterol showed a small absolute reduction at week 12 (-0.2, 95% confidence interval [CI] -0.3 to -0.0; and -0.2, 95% CI -0.3 to -0.0, respectively). Concentrations of ADMA, triglycerides, lipoprotein(a) and CRP did not change significantly. Analysis of covariance over the 12-week study period revealed no significant between-group differences for all parameters. No significant correlations were found between the concentrations of isoflavones and the CHD risk markers investigated.

**Conclusion:** Twelve-week administration of a supplement containing soy isoflavones and *Actaea racemosa* L. had little or no influence on the CHD risk markers studied. This supplement probably has neither protective nor adverse effects on the cardiovascular system, however, large long-term studies are needed to confirm this.

Marieke O. Verhoeven, Tom Teerlink, Peter Kenemans, Sonja D. Zuijdgeest-van Leeuwen, Marius J. van der Mooren

Fertility and Sterility, *in press*
1. Introduction

Estrogens alone or combined with a progestogen are the first choice of treatment for women with disabling climacteric complaints, such as hot flushes and night sweats. However, large randomized controlled trials reported negative effects of long-term use of menopausal hormone therapy (HT). As a result of these publications and the subsequent media exposure, women have become hesitant to start or continue HT even though climacteric symptoms may seriously interfere with their quality of life.

Therefore, it is necessary to search for an alternative option to obtain climacteric symptom relief, with fewer disadvantages. Isoflavones are nutritional components, present in, e.g., soy foods, that in vitro bind to the estrogen receptor. This has led to the hypothesis that isoflavones might reduce climacteric symptoms. However, so far the results of several studies have been inconsistent. An extract of black cohosh (Actaea racemosa Linnaeus (formerly called Cimicifuga racemosa L.)) has been suggested to reduce climacteric symptoms as well and is another potential alternative for HT.

The modulation of cardiovascular risk markers by estrogens alone or combined with a progestogen has been studied widely. Favorable effects of HT on the lipid profile are well documented. An emerging cardiovascular risk marker is asymmetric dimethylarginine (ADMA), an endogenous nitric oxide synthase (NOS) inhibitor. High levels of ADMA have been associated with increased overall mortality and cardiovascular events in specific patients groups. Concentrations ADMA were reduced by HT given orally (8%) or transdermally (4%).

Another cardiovascular risk factor is C-reactive protein (CRP), a marker for inflammation that is associated with coronary heart disease (CHD). Oral HT has been found to increase CRP in postmenopausal women, whereas transdermal HT did not.

Eight weeks’ consumption of fruit cereal bars enriched with soy isoflavones did not affect ADMA concentrations in postmenopausal women. The reported effects of isoflavones on the lipid profile vary greatly, and supplements containing isoflavones have been found not to influence CRP concentrations. No data have been available about effects of Actaea racemosa L. on ADMA and CRP until now, and studies investigating effects on lipids are rare.

Previously, we reported on a 12-week randomized, placebo-controlled, double-blind, study of a combination of soy isoflavones and Actaea racemosa L., with climacteric symptoms as primary endpoint. As secondary end points we also studied the effects on plasma concentrations of ADMA, arginine, symmetric dimethylarginine (SDMA), and the calculated arginine/ADMA ratio as a marker for NOS activity. Furthermore, we studied the effects on serum concentrations of lipids (total cholesterol, low-density lipoprotein (LDL)-cholesterol, high-density lipoprotein (HDL)-cholesterol, triglycerides and lipoprotein(a) (Lp(a)) and CRP. Correlations between these CHD risk markers and the previously reported plasma concentrations of isoflavones after 12 weeks of supplementation were investigated as well.
2. Materials and methods

2.1. Subjects. Healthy menopausal women aged 45 to 65 years with at least five hot flushes per day were recruited from the outpatient clinics of eleven centers in The Netherlands, and through regional newspaper advertisements. Participants had to be amenorrhoic for at least 6 months and their serum FSH concentration had to be higher than 25 IU/L. Women were not allowed to use medication, supplements or diets interfering with the supplement ingredients or the CHD risk markers investigated. Other exclusion criteria were published earlier. Dietary and physical habits were not registered during the study period; however, women were asked to maintain their usual dietary and physical habits during study participation.

The study conformed to the principles outlined in the Declaration of Helsinki and was approved by the central Institutional Review Board and the local Institutional Review Board of each participating center. All participants gave written informed consent before study entry.

2.2. Study Design. Details of this randomized, placebo-controlled, and double-blind study were published previously. In short, eligible women were randomly assigned to either the placebo (n = 64) or the supplement (n = 60) group, using a computer-generated randomization schedule in blocks of six. For a period of 12 weeks, women took orally two soft gel capsules twice per day during or just after breakfast and the evening meal to enhance absorption by bowel activity.

Women in the supplement group thus received daily among others 125 mg soy extract (providing 50 mg isoflavones including 24 mg genistein and 21.5 mg daidzein) combined with 100 mg Actaea racemosa L. extract (providing 8 mg deoxyacetein), whereas the women in the placebo group received daily 2,000 mg olive oil. The supplement and placebo capsules were provided by Numico (Wageningen, The Netherlands), and were identical in appearance, smell and taste. Women were considered non-compliant if, after one of the supplementation periods of 6 weeks, they returned more than 20% of the number of soft gel capsules handed out during the previous visit.

2.3. Measurements. Venous blood samples, for the determination of the concentrations of ADMA, arginine, SDMA, total cholesterol, LDL- and HDL-cholesterol, triglycerides, Lp(a) and CRP were collected between 8:00 and 10:00 a.m. at the screening visit and during the last visit at week 12. Subjects had fasted and refrained from smoking for at least 12 hours and from consuming alcohol for more than 24 hours before blood sampling. After 20 minutes of rest, blood was collected with a Vacutainer system (Becton Dickinson, Meylan, France) in tubes containing tripotassium ethylenediaminetetraacetic acid (K<sub>3</sub>EDTA) (Becton Dickinson) for ADMA, arginine and SDMA measurements in plasma and in plain tubes (Becton Dickinson) for lipids, including Lp(a), and CRP measurements in serum. Plasma and serum were separated by centrifugation at 2,000g, plasma at 4°C and serum at 20°C and both for 30 minutes within 1 hour of collection and divided into aliquots, snap-frozen and stored at -80°C until analysis. All samples from individual patients were analyzed in the same analytical series for each parameter.

The ADMA, arginine, and SDMA concentrations were measured by high-performance liquid chromatography with fluorescence detection. The interassay coefficients of variation (CVs) were less than 3% for ADMA and arginine and less
than 4% for SDMA. For each participant the plasma arginine/ADMA ratio was calculated.

Serum lipid levels were measured with a Modular P system (Roche, Mannheim, Germany). For total cholesterol, HDL-cholesterol, and triglycerides, the following reagents were used: CHOD-PAP, HDL-C plus, and GPO-PAP, respectively (all by Roche). The interassay CVs were less than 3.7%. LDL-cholesterol was calculated using the Friedewald formula. Serum Lp(a) concentrations were measured with a standard commercially available one-step sandwich ELISA using Immunozym Lp(a) (Progen Biotechnik, Heidelberg, Germany). The intraassay CV for this ELISA was 3.3% and the interassay CV was 4.5%.

CRP was assayed using an in-house highly sensitive ELISA with a lower limit of detection of 0.01 mg/L. The intraassay CV was 2.4% and the interassay CV was 10.6%. Methods for the measurement of plasma concentrations of isoflavones were described previously.

### 2.4. Statistics

Statistical analysis was performed using the Statistical Package for the Social Sciences PC version 10.0.5 (SPSS, Chicago, IL). Baseline characteristics and CHD risk markers are given as mean and standard deviation when normally distributed, or as median (25th and 75th percentile) when the distribution was skewed. Analyses of (co)variance [AN(C)OVA], with the baseline value of the variable under consideration and with study center and systolic blood pressure as constant covariates, were used for comparisons between the groups. Systolic blood pressure was used as covariate because this was significantly different between the groups at baseline. Study center was used as covariate since differences between centers could also potentially confound the study results.

The percentage changes from baseline are given as mean and 95% confidence interval (CI) or as geometric mean if the percentage changes had a skewed distribution. A two-tailed $P < 0.05$ was accepted as the level of statistical significance.

Because CRP levels are strongly influenced by acute infections and other inflammatory conditions, we identified all women who reported an infection in the weeks before blood sampling. In addition, women with a CRP concentration above 10 mg/L, which is considered to be the lower threshold level for the existence of an acute inflammation, were identified. The CRP concentration was evaluated in a sub-population excluding these women (placebo: $n = 53$; supplement: $n = 53$).

In both the placebo and supplement groups 41% of the women previously used hormones and had a washout of 6 weeks or more. This might not be enough in regard with the variables tested and could confound the study results. The age range 45-65 years allowed early (5 or less years after menopause) as well as late menopausal women (more than 5 years after menopause) to participate in this study. In early menopausal women changes in the cardiovascular markers investigated might differ from changes seen in late menopausal women. Therefore, the ANCOVAs were repeated with previous-hormone-use (yes or no) and with early or late menopausal as constant covariates.

Correlations were investigated between the CHD markers and the serum isoflavones concentrations by calculating the Pearson’s correlation coefficient in the supplement group. For these post-hoc analyses the Bonferroni correction for multiple comparisons was applied. The accepted significance level here was $P = .05/30 = .002$. 

3. Results

The study started in June 2002, and during a 5-month enrolment period, 173 women were screened, of whom 124 women were eligible to be randomized. The last participant completed the study in February 2003. Sixty-four women were randomly allocated to the placebo group and 60 women to the supplement group. During the study, eight women dropped out: five in the placebo group and three in the supplement group; reasons for dropout were published earlier.37 Of these eight women, three women accepted an alternative close-out visit during which blood could be collected (placebo: n = 2; supplement: n = 1). We analyzed these blood samples as if taken at the end-of-study visit. A completers analysis excluding these women did not show different results (data not shown).

From the group of women who completed the study, no blood samples were obtained from two women at baseline (placebo: n = 1; supplement: n = 1) and from two women at week 12 (placebo: n = 1; supplement: n = 1). These four women were excluded from the analyses. In the blood samples of two women in the placebo group no ADMA, arginine and SDMA were measured and in another woman in the placebo group no lipids and CRP were measured. As a result ADMA, arginine and SDMA were measured in 113 women (placebo: n = 57; supplement: n = 56) and lipids and CRP in 114 women (placebo: n = 58; supplement: n = 56). Women with an infection reported in the weeks prior to blood sampling and a CRP concentration above 10 mg/L, were excluded for the CRP analysis. Therefore, CRP was evaluated in a sub-population of 106 women (placebo: n = 53; supplement: n = 53).

Demographic characteristics of the population presented here did not differ significantly from the original population of 124 women.37 At baseline, no statistically significant differences were found between the study groups in the demographic characteristics of subpopulation evaluated

<table>
<thead>
<tr>
<th></th>
<th>placebo</th>
<th>supplement</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>59</td>
<td>56</td>
</tr>
<tr>
<td>age (years)</td>
<td>53.8 ± 4.4</td>
<td>54.1 ± 4.6</td>
</tr>
<tr>
<td>time since menopause (years)</td>
<td>3 (1 to 7)</td>
<td>5 (2 to 9)</td>
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<tr>
<td>body mass index (kg/m²)</td>
<td>25.5 ± 2.8</td>
<td>25.8 ± 2.4</td>
</tr>
<tr>
<td>blood pressure systolic (mmHg)</td>
<td>126 ± 11</td>
<td>131 ± 15a</td>
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<tr>
<td></td>
<td>81 ± 7</td>
<td>83 ± 9</td>
</tr>
<tr>
<td>smoking n (%)</td>
<td>12 (20.3)</td>
<td>9 (16.1)</td>
</tr>
<tr>
<td>previous hormone use n (%)</td>
<td>24 (40.7)</td>
<td>23 (41.1)</td>
</tr>
<tr>
<td>serum FSH (IU/L)</td>
<td>80.5 ± 28.2</td>
<td>76.8 ± 23.5</td>
</tr>
</tbody>
</table>

Table 1: Values are given as mean ± standard deviation, as median (25th to 75th percentile), or as number (%). No significant differences in baseline characteristics were found between the groups except for systolic blood pressure. Supplement = soy isoflavones and Actaea racemosa L.-containing supplement; n = number of women; FSH = follicle-stimulating hormone.
a: Analysis of variance: \( P = .04 \).
6. EFFECTS OF ISOFLAVONES AND ACTAEA RACEMOSA L. ON ADMA

<table>
<thead>
<tr>
<th></th>
<th>baseline</th>
<th>week 12</th>
<th>ANCOVA(^a)</th>
<th>mean % change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ADMA (µmol/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>0.467 ± 0.060</td>
<td>0.467 ± 0.063</td>
<td>.58</td>
<td>0.08 (-1.68 to 1.84)</td>
</tr>
<tr>
<td>Supplement</td>
<td>0.464 ± 0.053</td>
<td>0.467 ± 0.052</td>
<td>.49</td>
<td>0.91 (-0.86 to 2.67)</td>
</tr>
<tr>
<td>(P) value (^b)</td>
<td>.40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Arginine (µmol/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>93.7 ± 18.0</td>
<td>95.8 ± 17.9</td>
<td>.31</td>
<td>3.8 (-1.3 to 8.8)</td>
</tr>
<tr>
<td>Supplement</td>
<td>92.8 ± 17.3</td>
<td>94.2 ± 20.0</td>
<td>.39</td>
<td>1.9 (-2.4 to 6.3)</td>
</tr>
<tr>
<td>(P) value (^b)</td>
<td>.44</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SDMA (µmol/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>0.539 ± 0.072</td>
<td>0.546 ± 0.064</td>
<td>.41</td>
<td>1.86 (-0.29 to 4.01)</td>
</tr>
<tr>
<td>Supplement</td>
<td>0.538 ± 0.070</td>
<td>0.539 ± 0.087</td>
<td>.88</td>
<td>0.22 (-2.34 to 2.78)</td>
</tr>
<tr>
<td>(P) value (^b)</td>
<td>.29</td>
<td></td>
<td></td>
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<tr>
<td><strong>Arginine/ADMA ratio</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>202 ± 38</td>
<td>207 ± 39</td>
<td>.30</td>
<td>0.5 (-3.3 to 4.7)</td>
</tr>
<tr>
<td>Supplement</td>
<td>202 ± 38</td>
<td>204 ± 47</td>
<td>.70</td>
<td>-4.5 (-10.0 to 2.3)</td>
</tr>
</tbody>
</table>

Table 2: Concentrations are given as mean ± standard deviation. Percentage changes from baseline in ADMA, arginine and SDMA are given as mean (95% confidence interval) and in arginine/ADMA ratio as geometric mean (95% confidence interval) because this distribution was skewed. The number of women was 57 in the placebo group and 56 in the supplement group. Supplement = soy isoflavones and Actaea racemosa L.-containing supplement; ADMA = asymmetric dimethylarginine; SDMA = symmetric dimethylarginine

\(^a\): Analysis of covariance (ANCOVA) for between-group differences with the baseline value of the variable under consideration, study center, and systolic blood pressure at baseline as covariates.

\(^b\): ANCOVA, cross-sectional comparison for between-group differences with study center and systolic blood pressure at baseline as covariates.

characteristics (n = 115; Table 1), except for the systolic blood pressure (placebo: 126 ± 11 mmHg; supplement: 131 ± 15 mm Hg; ANOVA: \(P = .04\)). Three women (5.1%) in the placebo group and seven women (12.5%) in the supplement group used anti-hypertensive medication. This difference was not statistically significant (Fisher exact test: \(P = .20\)). Four women in the placebo group and one woman in the supplement group were noncompliant, leaving 93% of the women in the placebo group and 98% of the women in the supplement group compliant. Excluding these women from the analysis did not change the results (data not shown). The post hoc analysis with previous hormone use and early and late menopausal as constant covariates revealed no different results.

Table 2 and Figure 1 present the results of ADMA, arginine, and SDMA concentrations and the calculated arginine/ADMA ratio. Table 3 and Figures 2 and 3 present the results of lipids and CRP. The ANCOVA over the 12-week study period showed no significant differences in any of these variables between the placebo and the supplement group (Tables 2 and 3).

In the supplement group the absolute reduction in total and LDL-cholesterol was -0.2 (95% CI -0.3 to 0.0) and -0.2 (95% CI -0.3 to -0.0), respectively. The difference in percentage change from baseline between the placebo group and the supplement group was not statistically significant (placebo vs. supplement: total cholesterol, 2.0%, 95% CI 5.1 to 1.1%; \(P = .5\); LDL-cholesterol, -0.9%, 95% CI -5.3 to 3.6%; \(P = 1.0\); Fig. 2). HDL-cholesterol showed a significant increase from baseline in the placebo group (4.1%, 95% CI 0.5 to 7.6%). This increase did not significantly differ from the change seen in the supplement group (\(P = 0.07\)).
At baseline none of the women had detectable daidzein and genistein plasma concentrations. After 12 weeks, none of the women in the placebo group had detectable daidzein and genistein concentrations. In ten women in the supplement group daidzein and genistein concentrations were below the detection limits at week 12. These ten women were compliant according to the number of returned surplus capsules at the week 6 and 12 visits. Also in women that showed increased isoflavones concentrations in the supplement group at week 12 (n = 46) the ANCOVA showed no effects on CHD risk markers compared with baseline and the placebo group (data not shown).

In the whole supplement group (n = 56), the absolute values of daidzein and of genistein concentrations at week 12 did not correlate with the absolute concentrations or with the absolute and percentage changes of any of the CHD risk markers studied (data not shown). The same was observed when the ten women with no detectable daidzein and genistein concentration were excluded (n = 46, data not shown).
6. EFFECTS OF ISOFLAVONES AND ACTAEA RACEMOSA L. ON ADMA

Figure 2. Mean and 95% confidence interval (error bars) of the individual percentage changes from baseline in serum total cholesterol, LDL- and HDL-cholesterol, and geometric mean and 95% CI (error bars) of triglycerides and Lp(a) in the two groups after the 12-week supplementation period. supplement = soy isoflavones and Actaea racemosa L.-containing supplement; LDL = low-density lipoprotein; HDL = high-density lipoprotein; Lp(a) = lipoprotein(a).

4. Discussion

In the present study, the supplement tested, containing a combination of soy isoflavones extract and Actaea racemosa L., did not have any effect on measured blood concentrations of ADMA, arginine, SDMA, HDL-cholesterol, triglycerides, Lp(a) and CRP. The mean absolute reduction from baseline in total and LDL-cholesterol was significant in the supplement group, although not significantly different from the change seen in the placebo group. The HDL-cholesterol concentrations increased significantly in the placebo group, although the difference in changes between the two groups was not significant.

These results were not different from those in the completers analysis (placebo: n = 57; supplement: n = 55) or in the analysis excluding the women who were not compliant (placebo: n = 55; supplement: n = 55). Excluding the women in the supplement group with no detectable isoflavones concentrations at week 12 (placebo: n = 59; supplement: n = 46) or correcting for previous hormone use and early or late menopausal status did not modify the results.

A decrease in ADMA concentration has been observed during oral HT and it has been speculated that this could be a key phenomenon in the modulation of NO synthesis by HT. This could also be true for isoflavones, because they have estrogen receptor-binding characteristics. However, both the study by Reimann et al and the present study do not support this idea. The isoflavone genistean but
Concentrations of lipids and CRP

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Week 12</th>
<th>ANCOVA</th>
<th>Mean % change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total cholesterol</strong> (mmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>6.5 ± 1.0</td>
<td>6.4 ± 1.0</td>
<td>.20</td>
<td>-0.9 (-2.2 to 2.2)</td>
</tr>
<tr>
<td>Supplement</td>
<td>6.4 ± 1.1</td>
<td>6.3 ± 0.9</td>
<td>.27</td>
<td>-2.1 (-4.3 to 0.2)</td>
</tr>
<tr>
<td><strong>LDL-cholesterol</strong> (mmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>4.2 ± 0.9</td>
<td>4.1 ± 1.0</td>
<td>.45</td>
<td>-1.8 (-4.9 to 1.3)</td>
</tr>
<tr>
<td>Supplement</td>
<td>4.1 ± 1.0</td>
<td>3.9 ± 0.9</td>
<td>.13</td>
<td>-2.6 (-5.9 to 0.6)</td>
</tr>
<tr>
<td><strong>HDL-cholesterol</strong> (mmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>1.64 ± 0.36</td>
<td>1.74 ± 0.46</td>
<td>.06</td>
<td>4.07 (0.51 to 7.64)</td>
</tr>
<tr>
<td>Supplement</td>
<td>1.76 ± 0.45</td>
<td>1.70 ± 0.42</td>
<td>.11</td>
<td>-.73 (-3.03 to 1.57)</td>
</tr>
<tr>
<td><strong>triglycerides</strong> (mmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>1.3 (0.9 to 1.6)</td>
<td>1.3 (0.8 to 1.8)</td>
<td>.98</td>
<td>-0.8 (-8.8 to 7.8)</td>
</tr>
<tr>
<td>Supplement</td>
<td>1.1 (0.8 to 1.5)</td>
<td>1.1 (0.8 to 1.7)</td>
<td>.14</td>
<td>-0.3 (-7.4 to 7.3)</td>
</tr>
<tr>
<td><strong>Lp(a)</strong> (mg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>133 (61 to 330)</td>
<td>126 (59 to 297)</td>
<td>.54</td>
<td>-4.5 (-8.9 to 0.1)</td>
</tr>
<tr>
<td>Supplement</td>
<td>109 (62 to 213)</td>
<td>103 (61 to 221)</td>
<td>.13</td>
<td>-2.9 (-8.2 to 2.7)</td>
</tr>
<tr>
<td><strong>CRP</strong> (mg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>1.06 (0.63 to 2.19)</td>
<td>1.08 (0.66 to 2.01)</td>
<td>.68</td>
<td>-4.65 (-23.63 to 19.04)</td>
</tr>
<tr>
<td>Supplement</td>
<td>1.22 (0.62 to 1.76)</td>
<td>1.17 (0.57 to 2.39)</td>
<td>.76</td>
<td>4.05 (-10.30 to 20.67)</td>
</tr>
</tbody>
</table>

Table 3: Concentrations are given as mean ± standard deviation, as median (25th to 75th percentile). Percentage change from baseline in total cholesterol, LDL- and HDL-cholesterol are given as mean (95% confidence interval) and in triglycerides, Lp(a) and CRP as geometric mean (95% confidence interval) since these distributions were skewed. The number of women was 58 in the placebo group and 56 in the supplement group. CRP was evaluated in a subpopulation excluding women with a CRP above 10.0 mg/L or having an inflammatory disease during blood sampling (placebo: n = 53; supplement: n = 53). supplement = soy isoflavones and Actaea racemosa L.-containing supplement; LDL = low-density lipoprotein; HDL = high-density lipoprotein; Lp(a) = lipoprotein(a); CRP = C-reactive protein.

a: Analysis of covariance (ANCOVA) for between-group differences with the baseline value of the variable under consideration, study center, and systolic blood pressure at baseline as covariates.
b: ANCOVA cross-sectional comparison for between-group differences with study center and systolic blood pressure at baseline as covariates.

not daidzein has been found to induce an NO-dependent vasodilatation.\textsuperscript{41,42} An increased vasodilatation was observed after 6 months of supplementation with a relatively high dose of 54 mg genistean per day.\textsuperscript{42} This may in part explain the lack of change in ADMA in the present study, where 50 mg of isoflavones, providing only 24 mg genistean per day, was used for 12 weeks.

A meta-analysis of the effects of soy protein intake concluded that complete soy products reduce total cholesterol, LDL-cholesterol and triglycerides.\textsuperscript{43} It was hypothesized that the lipid-lowering effect of the complete soy product was mostly due to the isoflavones component of soy. This is not supported by several studies, including the present study, in which an extract containing only the isoflavones of
soy was used.\textsuperscript{44,45} Possibly, there is another component present in the total soy product that, alone or in combination with isoflavones, is responsible for the lipid-lowering effects.

During oral HT, an increase in CRP has been observed and it has been speculated that a HT-induced increase in CRP could have contributed to the increase in coronary events observed in the Heart and Estrogen/progestin Replacement Study.\textsuperscript{2,46} Soy isoflavones do not increase CRP and there appears to be no difference in effects whether the soy isoflavones are administered as a diet using the total soy product or as a soy extract containing only isoflavones.\textsuperscript{33,35,47}
Another potential mechanism for the observed lack of effect on ADMA, lipids and CRP of the supplement may be that soy isoflavones and *Actaea racemosa L.* are competitive and have counteractive effects when used together in one supplement. This could be true if the active components of the *Actaea racemosa L.* extract bind to the estrogen receptors as isoflavones do. However, in vivo experiments have demonstrated that the active components of the *Actaea racemosa L.* extract are acting through the serotonin receptor and not through the estrogen receptor.\textsuperscript{48}

The strength of this study is the randomized, placebo-controlled, double-blind design. A limitation of this study could be the lack of data on dietary and physical activity habits before and during the study. Diet and physical activity habits, as well as possible changes in these habits, were not registered in this study, although both can influence lipid levels.\textsuperscript{49,50} Publications on their effects on ADMA and CRP levels are scarce.\textsuperscript{51–53} Because in this study no effect was observed in both groups on ADMA and CRP and no difference in effects between the two groups on lipids, it is very unlikely that diet and physical activity habits would have influenced the outcomes of this study.

Ten women in the supplement group had no detectable phytoestrogen concentrations at week 12. According to capsule count these women were compliant. It is possible that the undetectable serum phytoestrogens concentrations are a result of a specific absorption problem. It is possible that phytoestrogens have less or no effects in this part of the population. However, repeating the analysis without the women in the supplement group with undetectable phytoestrogen levels at week 12 did not change the results.

Lipid profiles can be influenced by replacing saturated fats by olive oil (monounsaturated fat) in the daily diets.\textsuperscript{54–56} This suggests that using olive oil as a placebo for studying effects on lipids is inadequate and this can explain the increase in the HDL-cholesterol concentration in the placebo group. However, this cannot explain the lack of effect found in the supplement group. Studies investigating the effect of olive oil used larger amounts of olive oil that replaced the saturated fats usually consumed in the normal diet\textsuperscript{54–56} whereas in present study only 2 g of olive oil was supplemented while maintaining the usual diet habits. No effects were found on the other lipids apart from HDL-cholesterol. This makes it very unlikely that the use of olive oil as placebo has influenced the outcomes.

Other possible explanations for finding no effects are that the period of supplementation was too short for affecting the CHD parameters investigated. A longer treatment period may have induced significant changes. Previous hormone use may have influenced the results, because the required wash-out period was only 6 weeks. Also the wide age range allowed both early and late menopausal women to be included in the study. However, post hoc analysis revealed no influence of either factors on the study results.

Another limitation could be the sample size chosen in this study. This would be important if clinically relevant effects were found that did not reach statistical significance. In this study, however, no or very small changes were observed, having no clinical implications. Increasing the sample size might make the effects statistically significant, but without increasing the clinical relevance.

In conclusion, a combination of a soy isoflavones extract and *Actaea racemosa L.* did not influence plasma concentrations of ADMA, arginine, SDMA, lipids and
CRP. Therefore, it is probable that this supplement has neither a protective nor a harmful effect on the cardiovascular system.

Acknowledgements

The authors thank T.E. Vogelvang, MD, PhD\(^1\) for logistic management; A. Bouman\(^1\), PhD; S. de Jong\(^1\) and G. de Vrij\(^2\) for performing laboratory analyses (\(^1\)VU University Medical Center, Amsterdam, \(^2\)Numico Research BV, Wageningen, The Netherlands); and all women participating in the study.

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CHAPTER 7

Effects of a combination of isoflavones and Actaea racemosa Linnaeus on climacteric symptoms in healthy symptomatic menopausal women: a 12-week randomized, placebo-controlled, double-blind study

Objective: To investigate the effects of a novel dietary supplement containing soy isoflavones and Actaea racemosa Linnaeus (formerly called Cimicifuga racemosa L.) on climacteric symptoms in healthy menopausal women.

Design: In a multi-center, randomized, placebo-controlled, double-blind study, 124 women experiencing at least five vasomotor symptoms per 24 hours, were randomized to receive daily either the phytoestrogen-containing supplement (n = 60) or placebo (n = 64) for twelve weeks. The modified Kupperman Index and Greene Climacteric Scale, a visual analogue scale designed to measure quality of life and the daily number and severity of hot flushes, was used in the screening period and in weeks 6 and 12. Changes in these scores from baseline were calculated.

Results: At week 6 and 12, all scores in both groups had improved compared to baseline, though the overall difference in scores between the groups was not statistically significant.

Conclusion: The supplement containing soy isoflavones and A racemosa L. had no statistically significant effect on climacteric symptoms in perimenopausal women experiencing at least five vasomotor symptoms per day.

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Menopause 2005; 12: 412-420
1. Introduction

In the Netherlands, 50% to 75% of women aged 45 to 60 years report climacteric symptoms.¹ Hormone therapy (HT) has been the first choice of treatment for climacteric symptoms.² However, ever since the publication of results from large randomized controlled trials, the use of HT has been debated.³,⁴ These studies reported a negative effect of long-term HT on breast cancer incidence and no positive effect on cardiovascular events. In these studies, however, women with climacteric symptoms either were not included or were discouraged to participate. The observational Million Women Study also reported an adverse effect of HT on breast cancer risk.⁵ As a result of these publications and the subsequent media exposure, women have become hesitant to start or continue the use of HT even though climacteric symptoms may seriously interfere with their quality-of-life. Therefore, we need to search for alternative treatment strategies with a profile that is more acceptable to these women.

Interest has increased in phytoestrogens as an alternative to HT. Among the phytoestrogens, isoflavones are an important subgroup. In vitro, they have been shown to bind to the estrogen receptors, inducing an activity much weaker than that of 17β-estradiol.⁶ These observations provide the background for the hypothesis that isoflavones may reduce climacteric symptoms and possibly cause fewer side effects compared with HT. Although many isoflavones studies have been conducted, the results have been inconsistent. Some placebo-controlled studies reported significant reductions in climacteric symptoms,⁷–¹⁰ whereas others found no effect when compared with placebo.¹¹–²⁰ Therefore, whether isoflavones are an alternative for climacteric symptoms alleviation remains unclear.

Another alternative treatment option for symptomatic climacteric women is an extract of the rhizome of black cohosh, Actaea racemosa Linnaeus (formerly called Cimicifuga racemosa L.). Three randomized, placebo-controlled, double-blind studies investigating the effect of A. racemosa L. on climacteric symptoms have been reported²¹–²³ of which only one reported a statistically significant benefit over placebo.²¹

There is still no consensus as to whether treatment with soy isoflavones or A. racemosa L. is an effective option for the management of climacteric symptoms. Effects of a combination of these two substances have not been reported. This 12-week randomized, placebo-controlled, double-blind, study was designed to investigate the efficacy of a combination of isoflavones and A. racemosa L. in the alleviation of climacteric symptoms in healthy perimenopausal women.

2. Methods

2.1. Subjects. Healthy perimenopausal women aged 45 to 65 years who experienced at least five hot flushes per day were recruited from the outpatient clinics of 11 centers in the Netherlands and through regional newspaper advertisements. Women were enrolled in the study from June 2002 until October 2002. Participants had to be amenorrheic for at least 6 months and their serum follicle-stimulating hormone (FSH) concentration had to be higher than 25 IU/L. Women without a uterus but with intact ovaries were included if they had undergone a hysterectomy.
2. METHODS

more than 6 months before inclusion. Participants were not allowed to use nutritional supplements containing the same ingredients as the study supplement, with the exception of calcium (not exceeding 500 mg per day). Women consuming a vegetarian, vegan, macrobiotic diet, or a diet that included an intake of soy products more than once a week were excluded from participation. Other exclusion criteria were uncontrolled hypertension, thyroid disease, diabetes mellitus, other concomitant diseases interfering with the study parameters, a history of hormone-dependent (gynecologic) cancer, drug and alcohol abuse, mental disorders, and abnormalities in renal and liver functions. Women already using HT or other treatments (medicine or supplements) to alleviate climacteric complaints had a washout of at least 6 weeks before entering the study.

The study conformed to the principles outlined in the Declaration of Helsinki and was approved by the central Institutional Review Board and the local institutional review board of each participating center. All participants gave written informed consent before study entry.

2.2. Study design. The objective of the study was to determine the effects on climacteric symptoms of a novel dietary supplement that contains soy isoflavones and A racemosa L. The primary outcome of the study was the change from baseline with supplement compared with placebo in the calculated modified Kupperman Index score after 12 weeks of supplementation. Secondary efficacy parameters were changes in: modified Kupperman Index after 6 weeks, Greene Climacteric Scale, frequency and severity of hot flushes, and quality of life (measured by a Visual Analogue Scale [VAS]) after 6 and 12 weeks of supplementation.

Women were randomly assigned to either the placebo (n = 64) or the supplement (n = 60) group, using a computer-generated randomization schedule in blocks of six. For a period of 12 weeks, women took two softgel capsules orally twice per day during or just after breakfast and the evening meal. Women in the supplement group thus received daily 125 mg soy extract (providing 50 mg isoflavones including 24 mg genistein and 21.5 mg daidzein), 1,500 mg evening primrose oil extract (providing 150 mg gamma linoleic acid), 100 mg A racemosa L. extract (providing 8 mg deoxyacetein), 200 mg calcium, 1.25 µg vitamin D, and 10 IU vitamin E, whereas the women in the placebo group received 2,000 mg olive oil daily. Numico (Wageningen, The Netherlands) manufactured the softgel capsules containing either the phytoestrogens or olive oil, which were identical in appearance, smell, and taste. At the visits after 6 and 12 weeks the women returned the surplus softgel capsules, which were counted for compliance evaluation. Women were considered noncompliant if, after one of the supplementation periods of 6 weeks they returned, more than 20% of the number of softgel capsules that were handed out during the previous visit.

During a 2-week screening period, vasomotor symptoms were recorded daily in a diary. If women recorded a mean of five or more flushes every 24 hours and met all the other inclusion criteria, they were enrolled in the study and randomized. After 6 and 12 weeks they visited the outpatient clinic for assessments. Vasomotor symptoms recorded in the diary on a daily basis were classified either as hot flushes during daytime or as night sweats, during the week before the visits at weeks 6 and 12 of supplementation. Women categorized the daytime hot flushes as mild (a sudden sense of warmth without transpiration), moderate (sudden sense of warmth with transpiration without disturbing daily activities), or severe (sudden sense of
warmth with transpiration and disturbing daily activities). This classification made it possible to distinguish different vasomotor symptoms categories, namely, total daily number of hot flushes plus night sweats (vasomotor symptoms); total number of hot flushes during daytime; total number of night sweats; daily hot flushes divided in the total number of mild, moderate, and severe hot flushes. Another category was created by calculating the mean severity score per hot flush during daytime (see “Data analysis”). The evening before each visit, the women completed the Greene Climacteric Scale. During the visits the investigators scored the modified Kupperman Index and the women completed the VAS measuring quality of life.

To determine the concentrations of the isoflavones daidzein and genistein, venous blood samples were collected between 8 and 10 AM at the screening visit and during the last visit at the end of week 12. Subjects had fasted and refrained from smoking for at least 12 hours and from consuming alcohol for at least 24 hours before blood sampling. Determination of daidzein and genistein concentrations was performed after enzymatic treatment of the sample; the aglycones were extracted with tert-butyl methyl ether. The concentrations of daidzein and genistein were determined by high-performance liquid chromatography using ultraviolet absorbance as detection. Concentrations of daidzein below 5 μg/L and of genistein below 10 μg/L could not be detected with this method. The intra- and interassay coefficients of variation were 3%. The two samples provided by each woman (the screening sample and the week-12 sample) were analyzed in one run.

2.3. Data analysis. Sample size calculation was based on estimated changes in the modified Kupperman Index after a 12-week supplementation period. To detect a difference of at least 4 units in the modified Kupperman Index between the supplement and placebo groups using a squared standard deviation of 41.8 with a power of 0.80 and a significance level of 0.05 for two-sided test, a sample size of 43 women per group was required. Taking into account a dropout rate of 30%, a sample size of 56 women per group would be sufficient to detect a significant difference between groups, resulting in a total number of 112 women. The sample size was adjusted in order to correct for the loss of degrees of freedom because of the multicenter setup of this study. Assuming the participation of eleven centers, a total sample size of 124 women was necessary to obtain the same statistical power.

The total scores of the different vasomotor symptom categories in one recording period were divided by the number of days recorded to calculate the mean numbers per day per woman. Adding up the severity of all flushes (mild = 1, moderate = 2, and severe = 3) in one recording period and dividing this result by the total number of hot flushes in the same period resulted in the mean severity score per hot flush. For each parameter, differences in change after 6 and 12 weeks of supplementation compared with baseline between the supplement and placebo groups were analyzed using analysis of covariance for repeated measurements with the participating center as factor and the baseline value of the variable under consideration as constant covariate. Differences between the two groups at baseline and in changes at the visits after 6 and 12 weeks were analyzed using analysis of variance with the participating center as factor.

Analyses were performed on the intention-to-treat population including only women having measurements at baseline and at least one measurement during supplement evaluation. We used the last-observation-carried-forward procedure for missing values at the last visit in five cases. A per-protocol analysis was done on
3. RESULTS

Women screened N = 173

Women randomized N = 124

Placebo N = 64

SUPPL N = 60

Completers N = 59

Early termination (N = 3):
- Protocol violation (N = 1)
- Severe flushes complaints (N = 1)
- Severe headache (N = 1)

Not randomized (N = 49):
- Withdrew consent (N = 1)
- Breastfeeding (N = 48)

Early termination (N = 3):
- Weight gain, edema (N = 1)
- Withdrew consent (N = 1)
- Difficulty taking capsules (N = 1)

Completers N = 57

Figure 1. Study design and flowchart N = number; V = visit; SUPPL = phytoestrogens containing supplement; ☎ = telephone call; shaded area = vasomotor symptom diary. Measurements at visit 2, 3 and 4: modified Kupperman Index, Greene Climacteric Scale, and Visual Analogue Scale for quality-of-life. Blood was drawn at visit 1 and 4.

The intention-to-treat population excluding the women who did not complete the study or had major protocol violations.

Several post-hoc comparisons were done, to explore potential subgroups that would benefit from supplementation. To correct for multiple comparisons, we applied the Bonferroni P correction.

Correlations were calculated with the Pearson correlation coefficient. Correlations among age, body mass index (BMI), FSH, time since menopause and the modified Kupperman Index score, the total Greene Climacteric Scale score, and the different vasomotor symptom categories at baseline were analyzed. The changes in the concentrations of daidzein and genistein were correlated with the changes in the modified Kupperman Index score, the total Greene Climacteric Scale score, the quality-of-life VAS, and the different vasomotor symptom categories at week 12. All statistical analyses were performed using the Statistical Package for the Social Sciences 10.0.5 (SPSS Inc., Chicago, IL).
Characteristics of the two groups at baseline

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>SUPPL</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>64</td>
<td>60</td>
</tr>
<tr>
<td>Age (years)</td>
<td>53.7 ± 4.8</td>
<td>54.0 ± 4.9</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>25.5 ± 2.9</td>
<td>25.4 ± 3.0</td>
</tr>
<tr>
<td>Blood pressure systolic (mmHg)</td>
<td>127 ± 13.8</td>
<td>131 ± 14.0</td>
</tr>
<tr>
<td>Blood pressure diastolic (mmHg)</td>
<td>81 ± 8.4</td>
<td>83 ± 8.5</td>
</tr>
<tr>
<td>Smoking (%)</td>
<td>13 (20.3)</td>
<td>10 (16.7)</td>
</tr>
<tr>
<td>Time since menopause (years)</td>
<td>3 (1-7)</td>
<td>5 (2-9)</td>
</tr>
<tr>
<td>Previous hormone usea (%)</td>
<td>27 (42.2)</td>
<td>24 (40.0)</td>
</tr>
<tr>
<td>Serum FSH (IU/L)</td>
<td>81.2 ± 28.4</td>
<td>76.8 ± 23.5</td>
</tr>
</tbody>
</table>

Table 1: Values are given as mean ± SD, as median (25th-75th percentile) or as number of women (%). No statistically significant difference between the two supplement groups in the baseline characteristics.

SUPPL, A phytoestrogen containing supplement; FSH, follicle-stimulating hormone. aAll women using hormone therapy recently had a wash-out period of at least six weeks.

3. Results

During the 5-month enrollment period, 173 women were screened, of whom 124 women were eligible to be randomized (Figure 1). Within a center, the women were randomly allocated to the placebo group (n = 64) or the supplement group (n = 60). Differences in baseline characteristics between the two groups were not statistically significant (Table 1). At baseline, age, BMI, time since menopause, and FSH concentration showed no statistically significant correlation with the modified Kupperman Index score, the total Greene Climacteric Scale score or the different vasomotor symptom categories (data not shown). During the study eight women dropped out, five in the placebo group and three in the supplement group. Reasons for dropping out are shown in Figure 1. Women were considered compliant if they took at least 80% of the soft gel capsules in the complete study. Seven women in the placebo group and two women in the supplement group were non-compliant leaving 89% of the women in the placebo group and 97% of the women in the supplement group compliant.

The results of the intention-to-treat analysis (N = 124) did not differ from the per-protocol population analysis (n = 74); therefore, only the results of the intention-to-treat analysis are shown. Compared with baseline, the primary endpoint (the modified Kupperman Index score after 12 weeks) and the secondary endpoints (the Greene Climacteric Scale score as well as the vasomotor symptom categories) revealed a statistically significant reduction in both the placebo and the supplement group after 6 and 12 weeks of supplementation (Table 2). The magnitude of these reductions did not differ statistically significantly between the groups. The VAS for quality of life did not show statistically significant changes from baseline at week 6 or week 12 in either groups.
To identify a subgroup of women that could benefit from the supplementation, post hoc analysis were done dividing the group according to several characteristics. Analyses of covariance comparing smokers versus nonsmokers and recent HT users versus non-HT users were performed. In none of these groups was the supplement more beneficial than placebo in reducing climacteric symptoms. Other dichotomizations of the group were done using mean BMI (25.5 kg/m$^2$), mean VAS (72.3 mm), mean number of vasomotor symptoms per day (10.4), and the median of the number of years since menopause (4 years). Women who were above the mean or median were compared with those below the mean or median. These post-hoc analyses failed to reveal a characteristic by which women who might have benefited from taking the supplement over placebo could be identified. Additional post hoc analyses did not show a statistically significant trend in reducing vasomotor symptoms in a subgroup of women having at least nine vasomotor symptoms per day in the screening period. Among these women, the percentage reductions in total daily number and severity of hot flushes in the supplement group after 12 weeks (-51% and -14%, respectively) tended to be greater than those observed in the placebo group (-34% and -4%, respectively; $P > 0.5$ for both outcomes).

At baseline, the plasma samples of six women were not available for the determination of daidzein and genistein concentrations (placebo: $n = 5$; supplement: $n = 1$); at week 12, this was the case for seven women (placebo: $n = 4$; supplement: $n = 3$). At baseline, none of the women in the study had detectable daidzein and genistein plasma concentrations. None of the women in the placebo group had detectable daidzein and genistein concentrations after 12 weeks of supplementation. In 10 women of the supplement group, daidzein and genistein concentrations were below the detection limits. These 10 women were compliant according to the number of returned surplus softgel capsules at visits three and four. Without these 10 women, the median (25th to 75th percentile) of the concentrations of daidzein and genistein after twelve weeks of supplementation in the supplement group ($n = 47$) were 45 $\mu$g/L (29 - 72 $\mu$g/L) and 124 $\mu$g/L (78 - 191 $\mu$g/L), respectively.

In the group of women with detectable isoflavone concentrations, some symptom scores were significantly correlated with measured concentrations of daidzein and genistein. The absolute change after 12 weeks in the modified Kupperman Index correlated positively with the daidzein and genistein concentrations ($r = 0.33$, $P = 0.03$; $r = 0.40$, $P = 0.01$; respectively). The percentage change in the modified Kupperman Index correlated positively with the genistein concentration ($r = 0.33$, $P = 0.02$). The absolute ($r = 0.36$, $P = 0.01$; $r = 0.20$, $P = 0.18$; respectively) and percentage ($r = 0.38$, $P = 0.01$; $r = 0.26$, $P = 0.09$; respectively) change in the total Greene Climacteric Scale score correlated positively with the genistein concentration but not with the daidzein concentration at week 12. The absolute change in the total daily number of vasomotor symptoms at week 12 correlated with the genistein concentration ($r = 0.34$, $P = 0.02$). The number of different adverse events, including gastrointestinal problems, was similarly divided between the placebo and the supplement groups. No apparent clinically relevant changes in the tests of liver and kidney function were observed in either group.

4. Discussion

In the present study, the supplement was not effective in alleviating hot flushes in healthy symptomatic menopausal women experiencing at least five vasomotor
ISOFLAVONES, ACTAEA RACEMOSA L., AND CLIMACTERIC SYMPTOMS

**Table 2:** Values are given as mean and standard deviations, median (25th to 75th percentile), or mean percentage change [95% CI]. SUPPL, phytoestrogen-containing supplement.

<table>
<thead>
<tr>
<th>Item</th>
<th>Group</th>
<th>Baseline</th>
<th>Week 6</th>
<th>Week 12</th>
<th>P-value&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Percentage change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Modified Kupperman Index score</strong></td>
<td>Placebo</td>
<td>27.9 ± 5.8</td>
<td>22.5 ± 8.2</td>
<td>20.6 ± 8.8</td>
<td>0.65</td>
<td>-26 [-33 to -18]</td>
</tr>
<tr>
<td></td>
<td>SUPPL</td>
<td>28.1 ± 5.7</td>
<td>23.8 ± 6.9</td>
<td>20.7 ± 8.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.98</td>
<td>0.88</td>
</tr>
<tr>
<td><strong>Total score of Greene Climaacteric Scale</strong></td>
<td>Placebo</td>
<td>19.5 ± 7.3</td>
<td>14.8 ± 8.4</td>
<td>13.7 ± 7.0</td>
<td>0.50</td>
<td>-25 [-33 to -17]</td>
</tr>
<tr>
<td></td>
<td>SUPPL</td>
<td>18.2 ± 8.7</td>
<td>15.2 ± 7.7</td>
<td>13.3 ± 6.9</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.36</td>
<td>0.75</td>
</tr>
<tr>
<td><strong>Quality-of-life VAS (mm)</strong></td>
<td>Placebo</td>
<td>71.0 ± 16.0</td>
<td>68.8 ± 17.2</td>
<td>72.6 ± 15.6</td>
<td>0.54</td>
<td>-0.4 [-7 to 6]</td>
</tr>
<tr>
<td></td>
<td>SUPPL</td>
<td>73.8 ± 15.0</td>
<td>72.8 ± 18.5</td>
<td>76.3 ± 15.2</td>
<td></td>
<td>4 [-2 to 10]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.37</td>
<td>0.58</td>
</tr>
<tr>
<td><strong>Total number of vasomotor symptoms per 24 hours</strong></td>
<td>Placebo</td>
<td>10.5 (7.7-13.6)</td>
<td>6.7 (3.3-11.5)</td>
<td>6.4 (2.8-9.2)</td>
<td>0.94</td>
<td>-40 [-50 to -30]</td>
</tr>
<tr>
<td></td>
<td>SUPPL</td>
<td>10.7 (8.7-14.6)</td>
<td>8.1 (4.9-10.8)</td>
<td>5.9 (3.2-10.4)</td>
<td></td>
<td>-41 [-52 to -31]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.28</td>
<td>0.76</td>
</tr>
<tr>
<td><strong>Number of mild hot flushes during daytime</strong></td>
<td>Placebo</td>
<td>1.6 (0.0-8.4)</td>
<td>0.9 (0.2-2.2)</td>
<td>1.1 (0.2-2.1)</td>
<td>0.82</td>
<td>11 [-47 to 70]</td>
</tr>
<tr>
<td></td>
<td>SUPPL</td>
<td>1.5 (0.6-3.4)</td>
<td>1.0 (0.0-2.0)</td>
<td>0.7 (0.0-2.1)</td>
<td></td>
<td>59 [-95 to 213]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.52</td>
<td>0.76</td>
</tr>
<tr>
<td><strong>Number of moderate hot flushes during daytime</strong></td>
<td>Placebo</td>
<td>4.5 (2.4-6.4)</td>
<td>3.2 (0.6-4.8)</td>
<td>2.3 (0.6-4.3)</td>
<td>0.28</td>
<td>-37 [-53 to -21]</td>
</tr>
<tr>
<td></td>
<td>SUPPL</td>
<td>4.0 (2.7-7.8)</td>
<td>2.4 (1.1-4.7)</td>
<td>1.4 (0.2-4.3)</td>
<td></td>
<td>-47 [-61 to -33]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.44</td>
<td>0.16</td>
</tr>
<tr>
<td><strong>Number of severe hot flushes during daytime</strong></td>
<td>Placebo</td>
<td>1.1 (0.1-2.4)</td>
<td>0.0 (0.0-1.3)</td>
<td>0.0 (0.0-0.3)</td>
<td>0.69</td>
<td>-47 [-95 to 2]</td>
</tr>
<tr>
<td></td>
<td>SUPPL</td>
<td>0.8 (0.0-2.9)</td>
<td>0.0 (0.0-0.8)</td>
<td>0.0 (0.0-1.0)</td>
<td></td>
<td>-40 [-75 to -6]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.64</td>
<td>0.48</td>
</tr>
<tr>
<td><strong>Mean severity score per hot flush</strong></td>
<td>Placebo</td>
<td>1.9 ± 0.4</td>
<td>1.7 ± 0.7</td>
<td>1.7 ± 0.6</td>
<td>0.71</td>
<td>-5 [-18 to -5]</td>
</tr>
<tr>
<td></td>
<td>SUPPL</td>
<td>1.9 ± 0.4</td>
<td>1.7 ± 0.6</td>
<td>1.6 ± 0.7</td>
<td></td>
<td>-19 [-28 to -11]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.97</td>
<td>0.14</td>
</tr>
</tbody>
</table>

<sup>a</sup>Analysis of covariance for repeated measurements with participating center as factor and the baseline value of the variable under consideration as constant covariate (treatment effect).

<sup>b</sup>Analysis of variance for between-group comparison with participating center as factor. Mean percentage change [95% CI] from baseline at week 12.
reductions in the placebo group to be as high as 20% to 50%. In the present study, the reduction in total number of daily vasomotor symptoms in the placebo group was 36%. The studies reporting no or a small placebo effect were often able to detect an additional effect from the isoflavones when compared with placebo. This large variation in placebo effect underlines the importance of the placebo-controlled design. However, even among placebo-controlled studies, results are difficult to compare.

A single-blind run-in period with a placebo in both groups may reduce the initial effect of a placebo. In a placebo-controlled study using a red-clover isoflavones dietary supplement (80 mg isoflavones: daidzein, genistein, formonetin and biochanin A), the double-blind supplementation period of 12 weeks was preceded by a single-blind, 4-week run-in period in which all women received placebo. During the run-in period, the number of hot flushes decreased by 16.7% in the total group of women. During the subsequent 12-week supplementation phase, no further reduction was found in the placebo group, whereas the isoflavones group showed an additional 44% reduction in the number of hot flushes.

In most studies, women took supplements for 12 weeks. In the majority of these studies, the placebo group showed a progressive reduction until week 12. Therefore, the effect of a supplementation period longer than 12 weeks is uncertain, for both phytoestrogens and placebo. The effect of isoflavones on climacteric symptoms might be sustained, whereas the placebo effect might disappear. In one randomized, placebo-controlled, double-blind study, women’s diets were supplemented for 24 weeks. These women were randomly assigned to three groups to receive an isoflavone-rich soy protein diet, or an isoflavone-poor soy protein diet, or a whey protein diet. After 12 weeks, the decrease in hot flushes in all three groups was significant compared with baseline but not significantly different among the groups. Interestingly, after 24 weeks the effect had disappeared in the isoflavone-poor soy protein diet group whereas it was sustained in the other two groups. This finding indicates not only that 12-week supplementation may be too short a period, but also that longer-term studies are needed to prove the effects of isoflavones beyond the placebo effect. Moreover, even after 24 weeks, the decrease in symptoms in the group receiving a whey protein diet was still the same as that observed in the isoflavone-rich group. This finding suggests that placebo effects can last longer than 24 weeks. Therefore, to differentiate between the effects of phytoestrogen and placebo, the study duration should be increased substantially to periods that are practiced in real life.

Another explanation for the lack of an additional effect of supplementation in the present study could be the low isoflavones dose in this particular supplement. However, some other studies that used higher doses of isoflavones also did not find an effect on climacteric symptoms, though some studies that used lower doses of isoflavones did find a significant effect on climacteric symptoms. In the present study, the dose of A racemosa L. was higher compared with that used in other studies. In contrast to our study, two randomized, placebo-controlled, double-blind studies reported a positive effect of A racemosa L. on climacteric symptoms measured by the Menopause Rating Scale and the Kupperman Index. Both studies used a lower dose of A racemosa L.. A possible explanation for the contrasting observation may be the difference in the number of vasomotor symptoms
in the populations studied. The mean number of vasomotor symptoms at baseline in the study by Stoll\textsuperscript{21} was five, whereas in our study the mean number was ten.

Three clinical trials compared the effect on climacteric symptoms of isoflavone-poor soy protein and isoflavone-rich soy protein.\textsuperscript{16,18,27} None of these studies reported a difference in the alleviation of climacteric symptoms. Burke et al\textsuperscript{27} and Han et al\textsuperscript{18} used isoflavone-poor soy protein as a control. If there is another component in the soy protein that could alleviate climacteric symptoms, this could explain the lack of a difference in effect on climacteric symptoms. St. Germain et al\textsuperscript{16} used a third group that was supplemented with whey seed. The authors found no beneficial effect of isoflavone-rich soy protein over isoflavone-poor soy protein or whey seed.

A potential mechanism for the observed lack of effect on climacteric symptoms in the supplement group may be that soy isoflavones and \textit{A racemosa L.} are competitive and counteract with each other when used together in one supplement. This could be true if the active components of the \textit{A racemosa L.} extract bind to the estrogen receptors as the isoflavones do. However, in vivo experiments have pointed out that active components of the \textit{A racemosa L.} extract are acting through the serotonin receptor and not through the estrogen receptor.\textsuperscript{28}

In the present study, the supplement seemed to reduce the severity score of hot flushes in a subgroup of women experiencing at least nine vasomotor symptoms per day. An effect on severity of vasomotor symptoms was also found by Upmalis et al.\textsuperscript{14} Apparently, phytoestrogen-containing supplements are not always able to eliminate all symptoms, but they may offer relief, especially to those women who suffer most. This was confirmed by Messina et al,\textsuperscript{29} who found a positive correlation between baseline hot flush frequency and treatment efficacy ($r = 0.68; P = 0.01$).

Two studies found a larger number of hot flushes in women with a high BMI compared with women with a low BMI in pre- and perimenopausal women, not in postmenopausal women.\textsuperscript{30,31} In our study, women had to be amenorrheic for at least 6 months, so some of them were in the perimenopausal phase in which BMI can influence the presence of climacteric symptoms. In a pos hoc analysis comparing women with a high and low BMI, a modulating influence of BMI on the efficacy of the supplement was excluded. In addition, a post hoc analysis was done comparing women with a high and a low VAS score, because it was thought that general well-being could influence the effect of supplementation on the number and severity of climacteric symptoms. However, this post hoc analysis also revealed no significant difference.

The strength of this randomized, placebo-controlled, double-blind study is its design. The number of women in the study was based on a predetermined sample-size. A limitation of this study is that there was no run-in period for excluding a possible placebo effect. A second limitation of this study is the short supplementation period. Although the number of women in the subgroup with nine vasomotor symptoms or more per day was 81, the power for detecting statistically significant differences between placebo and supplement in this subgroup was too low.

Women with five vasomotor symptoms per day were included in this study. This is different from the Food and Drug Administration’s criterion of at least eight moderate to severe vasomotor symptoms per day, but is comparable with many other phytoestrogen studies.\textsuperscript{11,15,16,25} After all, phytoestrogens are not intended to be used as a pharmacological therapy, though they may offer symptom
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relief, especially if symptoms are classified as moderate. For the same reason, we also decided not to adhere to the Food and Drug Administration’s guidelines for frequency of outcome measurements.

Another possible limitation of the present study could be our choice of the primary endpoint, the modified Kupperman Index. Alder criticized the Kupperman Index for its weighting of the symptoms by different factors. Some symptoms were considered more important than others and are multiplied by these factors. However, there is no clear justification for the chosen multiplication factor. Therefore, the Greene Climacteric Scale was also used in this study. The total scores of both scales were significantly reduced after 12 weeks of supplementation, and the changes in scores on both instruments in the placebo and supplement groups were not significantly different.

5. Conclusion

A novel dietary supplement given for 12 weeks did not improve climacteric symptoms in perimenopausal women experiencing at least five vasomotor symptoms per day.

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References


CHAPTER 8

Effects on asymmetric dimethylarginine of HMR 3339, a novel selective estrogen receptor modulator: a 12-week, randomized, placebo-controlled, double-blind, dose-ranging study in healthy postmenopausal women

OBJECTIVE: To investigate the short-term effects of three different doses of the selective estrogen receptor modulator HMR 3339 in comparison with placebo and raloxifene on asymmetric dimethylarginine (ADMA), a nitric oxide synthase inhibitor.

DESIGN: This study was a multi-center, randomized, placebo-controlled, double-blind, dose-ranging study. Ninety-four healthy postmenopausal women received daily doses of either placebo (n = 16), HMR 3339 2.5 mg (n = 20), HMR 3339 10 mg (n = 19), HMR 3339 50 mg (n = 20), or raloxifene 60 mg (n = 19) for 12 weeks. Fasting plasma concentrations of ADMA, arginine, and symmetric dimethylarginine (SDMA) were measured at baseline and after 4 and 12 weeks by high-performance liquid chromatography.

RESULTS: HMR 3339 induced a dose-dependent reduction of ADMA and SDMA concentrations, with the largest effects (P < 0.01 for both) in the HMR 3339 50 mg group compared with baseline and placebo (at 12 weeks: -7.0% [95% CI, -14.2% to 0.2%] for ADMA and -16.2% [95% CI, -22.4% to -10.0%] for SDMA). Twelve weeks of raloxifene 60 mg significantly reduced SDMA (P = 0.03) but not ADMA concentrations. Arginine concentrations were not altered by any treatment.

CONCLUSIONS: The reduction of the nitric oxide synthase inhibitor ADMA by HMR 3339 may potentially have a beneficial effect on the cardiovascular system in postmenopausal women.

Marieke O. Verhoeven, Tom Teerlink, Peter Kenemans, Tatjana E. Vogelvang, Marius J. van der Mooren

Menopause, in press
1. Introduction

Coronary heart disease (CHD) is the leading cause of death in postmenopausal women in Western countries. The progressive increase in CHD after menopause suggests that estrogens are protective in premenopausal women and that postmenopausal hormone therapy (HT) could protect peri- and postmenopausal women from this increase in CHD. However, recently published randomized clinical trials reported no benefit, and even an early CHD harm, for elderly postmenopausal women with oral HT use. An adverse effect of combined HT on breast cancer risk was observed as well. Because of media exposure associated with these results, HT use has encountered much hesitancy, both from practitioners and from women with climacteric complaints. Therefore, it is necessary to search for alternatives for the treatment of climacteric complaints, preferably with beneficial effects on the long-term consequences of menopause, like the increased risk of CHD.

A possibility is that an alternative could be found among selective estrogen receptor modulators (SERMs). Raloxifene is a second-generation SERM that is marketed for the prevention and treatment of osteoporosis. Raloxifene has estrogen receptor agonistic effects on cardiovascular risk markers and bone as well as estrogen receptor antagonistic effects on the breast and uterus. It was also found to reduce the risk of cardiovascular events in osteoporotic postmenopausal women at high cardiovascular risk; however, this protective effect was not found after prolonging the treatment to 7.8 years. Conclusive data should come from the Raloxifene Use for The Heart study. Unfortunately, raloxifene does not alleviate, and may even exacerbate, vasomotor symptoms.

HMR 3339 (4-chloro-11β-(4-(2-(diethylamino)ethoxy)phenyl)-estra-1,3,5(10)- triene-3, 17β-diol) is a newly developed SERM that binds to the human recombinant estrogen receptor and exerts tissue-specific agonistic and antagonistic activity in in vitro and in vivo models. Preliminary data suggest positive effects on bone strength and a favorable safety profile on the breast and uterus. Furthermore, we have previously shown beneficial changes in some cardiovascular risk markers. HMR 3339 induced dose-dependent reductions of total and low-density lipoprotein cholesterol, C-reactive protein, homocysteine, fibrinogen and procarboxypeptidase U. Effects of HMR 3339 on luteinizing hormone concentrations have suggested potential beneficial effects on vasomotor symptoms.

Asymmetric dimethylarginine (ADMA) is an emerging CHD risk factor that inhibits nitric oxide synthase (NOS). High levels of ADMA have been associated with an increased overall mortality and more cardiovascular events in specific patient groups. ADMA is reduced by HT given orally and transdermally.

We hypothesized that, like oral estrogens, HMR 3339 reduces ADMA concentrations. Therefore, the aim of this study was to compare, in a randomized, placebo-controlled, double-blind study, the short-term effects of three doses of HMR 3339 and of raloxifene 60 mg on plasma concentrations of ADMA. Additionally, we measured changes in concentrations of the NOS substrate arginine and symmetric dimethylarginine (SDMA), a stereoisomer of ADMA that does not inhibit NOS.
2. Methods

2.1. Subjects and study design. The design of this study has been published previously. The investigation conformed to the principles outlined in the Declaration of Helsinki. The institutional review boards of all participating centers approved the protocol. Written informed consent was obtained from each participant before entry into the study.

Healthy, postmenopausal women aged 50 to 65 years with no history of hysterectomy were recruited from the outpatient clinics of eight hospitals in The Netherlands and one clinic in Belgium, and through regional newspaper advertisements. Participants had a body mass index (BMI) between 18 and 30 kg/m², had had their last menstrual period between 2 and 10 years before entering the study, did not smoke more than 15 cigarettes per day, and had fewer than five vasomotor symptoms per week. At screening, follicle-stimulating hormone concentration had to be above 20 IU/L.

Exclusion criteria included a history of cardiovascular, venous thromboembolic, metabolic, or endocrinologic diseases; estrogen-dependent neoplasia; severe endometriosis; excess consumption of alcohol or abuse of drugs; and clinically relevant abnormalities in laboratory tests of renal and hepatic function. Women also were excluded if they had participated in a clinical trial with investigational drugs in the 3 months preceding the study or in another HMR 3339 study. During the study women were prohibited from using any treatment for menopausal symptoms, HT for other purposes, treatment that might interfere with the coagulation profile, or lipid-lowering drugs.

Eligible women were randomly assigned to daily treatment with either placebo (placebo group), or HMR 3339 2.5 mg (Hoechst Marion Roussel R&D, Romainville, France), or HMR 3339 10 mg, or HMR 3339 50 mg, or raloxifene 60 mg (Eli Lilly and Co, Indianapolis, IN). All medications, including placebo and raloxifene 60 mg, were put into identical capsules to ensure double-blinding; these were taken orally. The study medication was packaged (by Hoechst Marion Roussel R&D) according to a random-number table and assigned to the participants in sequence by the investigators of the various centers. Women participating in the study, the investigators, and the person performing the laboratorial measurements were all blinded to study medication. The blinding was discontinued after all study parameters were collected and the database was locked.

2.2. Laboratory measurements. ADMA, arginine, and SDMA were determined at baseline and after 28 days (±3 days) and 84 days (±3 days) of treatment. The subjects had fasted and refrained from smoking for at least 12 hours and from consuming alcohol for more than 24 hours before blood sampling. After 20 minutes of rest, blood was collected with a Vacutainer system (Becton Dickinson, Meyren Cedex, France) into a cooled ethylenediaminetetraacetic acid tube. The blood samples were immediately placed in ice and centrifuged at 2,500 g at 4°C for 15 minutes within 1 hour of collection. 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. All samples from individual participants were analyzed in the same analytical series. The interassay coefficients of variation were less than 3% for ADMA and arginine and less than 4% for SDMA. For
each participant the plasma arginine/ADMA ratio was calculated. The analyses of follicle-stimulating hormone, lipids, homocysteine and C-reactive protein were described previously.\textsuperscript{14,15}

**2.3. Statistical analysis.** The sample size calculation was based on estimated changes in the primary endpoint, urinary collagen-C-telopeptides, after 12 weeks. To detect a difference of at least 21% change between the placebo and active treatment groups, using a standard deviation of 23% with a power of 0.80 and a significance level of 0.05 for two-sided test, a sample size of 20 women per group was required. As secondary endpoints ADMA, arginine, and SDMA concentrations were measured, and these results are reported here.

Statistical analysis was performed using the Statistical Package for the Social Sciences, version 12.0.1 (SPSS Inc, Chicago, IL). Values are given as mean ± SD. Standard parametric tests were used to compare baseline measurements and individual percentage changes from baseline between groups. Analysis of covariance (ANCOVA) for repeated measurements with the baseline value of the variable under consideration as a constant covariate was used for comparisons among and between the groups. A regression analysis was used to evaluate dose-dependent effects of the three HMR 3339 groups. A two-tailed $P$ value less than 0.05 was considered significant.

Data of 94 women were available for the intention-to-treat analysis for ADMA, arginine, and SDMA (placebo: $n = 16$; HMR 2.5: $n = 20$; HMR 10: $n = 19$; HMR 50: $n = 20$; and raloxifene 60: $n = 19$; Fig. 1). In this analysis the last-observation-carried-forward procedure was applied for missing results in six cases using the results obtained at the previous visit (placebo: $n = 1$; HMR 2.5: $n = 1$; HMR 10: $n = 1$; HMR 50: $n = 2$; and raloxifene 60: $n = 1$). In addition, we analyzed data from women who had a blood sample at all visits (completers analysis, $n = 88$). No differences between the intention-to-treat analysis and the completers analysis were found; therefore, only the data of the intention-to-treat analysis are shown.

Correlations of age, time since menopause, body mass index, blood pressure and follicle-stimulating hormone concentrations with baseline concentrations of ADMA were assessed by calculating Pearson’s correlation coefficient. Furthermore, correlations were calculated between baseline values of ADMA and baseline values of arginine, SDMA, and several other relevant factors measured previously in this study (total cholesterol, low-density and high-density lipoprotein cholesterol, triglycerides, homocysteine and C-reactive protein).\textsuperscript{14,15} Correlations between the absolute changes in ADMA and the absolute changes in these factors after 12 weeks of treatment were calculated in the three HMR 3339 groups and raloxifene group combined. For these post hoc analyses the Bonferroni $P$ correction for multiple comparisons was applied. Because 22 correlations were calculated, the accepted significance level here was $P = 0.002 (0.05/22)$.

**3. Results**

The study started in October 1999. During the enrolment period, 168 women were screened, of whom 118 women were eligible to be randomized. The last participants completed the study in September 2000. For logistic reasons, no blood was collected from 23 women for the measurements of ADMA, arginine, and SDMA (placebo group: $n = 6$; HMR 2.5 group: $n = 5$; HMR 10 group: $n = 4$; HMR
50 group: n = 4; raloxifene 60 group: n = 4). One woman (HMR 10 group) was excluded from the analysis because she had no blood sample at baseline. Six women discontinued the treatment before the end of the study: one woman in the placebo group because of an abnormal result on the liver function test (withdrawn on day 20); one woman in the HMR 2.5 group because of an abnormal finding on electrocardiography at baseline (withdrawn on day 6); one woman in the HMR 10 group because of menopausal complaints (withdrawn on day 52); two women in the HMR 50 group, one because of an abnormal result on the liver function test (withdrawn on day 12) and one woman because of hot flashes (withdrawn on day 67); and one woman in the raloxifene 60 group because of an abnormal result on the baseline liver function test (withdrawn on day 2). An additional blood sample was taken from the six women who withdrew, and we analyzed these blood samples as if they had been taken at the next scheduled visit. Two women (HMR 10 group: n = 1; HMR 50 group: n = 1) had no blood samples at week 4; however, these women were included in the analysis by using the last-observation-carried-forward procedure. This left 94 women (placebo group: n = 16; HMR 2.5 group: n = 20; HMR 10 group: n = 19; HMR 50 group: n = 20; and raloxifene group 60: n = 19) eligible for the current substudy.

**Figure 1.** Mean of the individual percentage changes from baseline and SEM (error bars) in asymmetric dimethylarginine (ADMA), arginine and symmetric dimethylarginine (SDMA) concentrations in the five study groups at week 12. 

*t* test for between-group differences: #0.10 > *P* > 0.05, *P* < 0.05, **P* < 0.01, ***P* < 0.001.

HMR 2.5, HMR 3339 2.5 mg; HMR 10, HMR 3339 10 mg; HMR 50, HMR 3339 50 mg; raloxifene, raloxifene 60 mg.
<table>
<thead>
<tr>
<th>Baseline characteristics</th>
<th>Placebo (n = 16)</th>
<th>HMR 2.5 (n = 20)</th>
<th>HMR 10 (n = 19)</th>
<th>HMR 50 (n = 20)</th>
<th>Raloxifene (n = 19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>55.1 ± 3.0</td>
<td>55.8 ± 3.2</td>
<td>55.7 ± 3.1</td>
<td>56.3 ± 4.4</td>
<td>54.8 ± 2.7</td>
</tr>
<tr>
<td>Duration of amenorrhoea, y</td>
<td>5.6 ± 2.5</td>
<td>4.9 ± 2.4</td>
<td>5.1 ± 2.5</td>
<td>5.6 ± 2.4</td>
<td>5.3 ± 2.1</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>24.8 ± 2.0</td>
<td>25.7 ± 2.6</td>
<td>25.0 ± 2.8</td>
<td>25.4 ± 3.3</td>
<td>24.5 ± 3.1</td>
</tr>
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<td>Blood pressure, mm Hg</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Systolic</td>
<td>131 ± 17</td>
<td>124 ± 17</td>
<td>126 ± 17</td>
<td>133 ± 15</td>
<td>125 ± 15</td>
</tr>
<tr>
<td>Diastolic</td>
<td>81 ± 10</td>
<td>79 ± 10</td>
<td>80 ± 10</td>
<td>83 ± 7</td>
<td>82 ± 11</td>
</tr>
<tr>
<td>Smokers, n (%</td>
<td>4 (25)</td>
<td>4 (20)</td>
<td>6 (32)</td>
<td>2 (10)</td>
<td>4 (21)</td>
</tr>
<tr>
<td>Serum FSH, IU/L</td>
<td>57.9 ± 21.9</td>
<td>55.0 ± 20.9</td>
<td>54.5 ± 17.5</td>
<td>59.5 ± 21.6</td>
<td>65.6 ± 17.0</td>
</tr>
<tr>
<td>Total cholesterol, mmol/l</td>
<td>6.2 ± 1.0</td>
<td>6.1 ± 0.8</td>
<td>6.1 ± 1.1</td>
<td>6.0 ± 1.1</td>
<td>6.0 ± 0.8</td>
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</tbody>
</table>

Table 1: Values are mean ± SD or number (percentage). HMR 2.5, HMR 3339 2.5 mg; HMR 10, HMR 3339 10 mg; HMR 50, HMR 3339 50 mg; Raloxifene, raloxifene 60 mg; FSH, follicle-stimulating hormone. There were no statistically significant differences between the five groups in the baseline characteristics.
4. DISCUSSION

Demographic characteristics of the population analyzed (n = 94) did not differ significantly from the original population of 118 women. At baseline no significant differences were found between the groups in the demographic characteristics (Table 1). The number of smokers was unequally distributed over the five groups, but statistical corrections for smoking as a factor in the ANCOVA model did not change the results (data not shown). None of the women randomized in the study used any treatment interfering with the coagulation profile, or lipid-lowering drugs before or during the study. Table 2 provides plasma concentrations of ADMA, arginine, SDMA and the calculated arginine/ADMA ratio at the different time points. Figure 1 shows the mean percentage changes of ADMA, arginine, and SDMA in all groups after 12 weeks of treatment. ANCOVA over the 12-week treatment period revealed significant changes in ADMA (P = 0.01). HMR 3339 showed dose-dependent changes in ADMA concentrations (P = 0.01), with the largest effect in the HMR 3339 50 group compared with baseline and placebo at week 12 (HMR 2.5: 0.0% [95% CI, -6.5% to 6.6%]; HMR 10: -1.3% [95% CI, -7.3% to 4.8%]; HMR 50: -7.0% [95% CI, -14.2% to 0.2%]). Twelve weeks of raloxifene 60 mg did not have any effect on ADMA concentrations compared with placebo. ANCOVA over the 12-week treatment period revealed no significant changes in arginine in any of the groups.

Although the baseline ADMA and arginine concentrations were not significantly different between any of the five groups, the baseline SDMA concentrations did differ, with the highest concentration in the HMR 50 group (Table 2). A dose-dependent reduction in SDMA concentrations with HMR 3339 was seen as well (P = 0.03), with the largest reduction in the HMR 50 mg group at week 12 (HMR 2.5: -8.8% [95% CI, -15.9% to -1.7%]; HMR 10: -11.7% [95% CI, -19.7% to -3.8%]; HMR 50: -16.2% [95% CI, -22.4% to -10.0%]). The ANCOVA showed a significant reduction in SDMA concentrations in the raloxifene group in comparison to baseline and placebo at week 12. However, this observation could be due to the increase in the placebo group compared with baseline (raloxifene compared with baseline and placebo: -7.2% [95% CI, -14.1% to -0.2%]; placebo compared to baseline: +7.7% [95% CI, 2.2% to 13.3%]; Table 2; Fig. 1). The arginine/ADMA ratio did not show any changes over the 12-week study period in the treatment groups compared with baseline. Baseline characteristics and baseline values of arginine, SDMA and the other parameters measured previously in this study did not correlate with baseline ADMA concentrations (data not shown). Only the absolute change from baseline in arginine concentrations was significantly correlated with the absolute change from baseline in ADMA concentrations at week 12 (r = 0.40, P < 0.001).

4. DISCUSSION

In this study 12 weeks of treatment with HMR 3339 50 mg, but not with raloxifene 60 mg, reduced ADMA concentrations. Arginine showed no significant changes in any of the treatment groups compared with baseline at week 12. In all active treatment groups, SDMA concentrations were reduced in comparison with the placebo group.

The average reduction in ADMA concentrations induced by the HMR 50 group versus placebo observed in this study (approximately 7%) is similar to the reduction found after oral treatment with conjugated equine estrogens as well as 17β-estradiol (approximately 7%) published previously by our group. Although
Concentrations of ADMA, arginine, SDMA, and the arginine/ADMA ratio during 12 weeks of treatment

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Week 4</th>
<th>Week 12</th>
<th>ANCOVA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ANCOVA&lt;sup&gt;b&lt;/sup&gt;</th>
<th>% Change Weeks 0-4&lt;sup&gt;c&lt;/sup&gt;</th>
<th>% Change Week 0-12&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ADMA, μmol/L</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>0.446 ± 0.046</td>
<td>0.461 ± 0.053</td>
<td>0.446 ± 0.040</td>
<td>0.96</td>
<td>-3.3 (-9.9 to 3.4)</td>
<td>0.0 (-6.5 to 6.6)</td>
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</tr>
<tr>
<td>HMR 2.5</td>
<td>0.462 ± 0.038</td>
<td>0.462 ± 0.045</td>
<td>0.467 ± 0.045</td>
<td>0.82</td>
<td>-1.6 (-7.0 to 3.8)</td>
<td>-1.3 (-7.3 to 4.8)</td>
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</tr>
<tr>
<td>HMR 10</td>
<td>0.452 ± 0.040</td>
<td>0.460 ± 0.038</td>
<td>0.451 ± 0.035</td>
<td>&lt; 0.01</td>
<td>-8.0 (-13.7 to 2.3)</td>
<td>-7.0 (-14.2 to 0.2)</td>
<td></td>
</tr>
<tr>
<td>HMR 50</td>
<td>0.459 ± 0.057</td>
<td>0.435 ± 0.040</td>
<td>0.431 ± 0.040</td>
<td>0.08</td>
<td>-4.3 (-10.0 to 1.5)</td>
<td>1.7 (-5.2 to 8.0)</td>
<td></td>
</tr>
<tr>
<td>Raloxifene</td>
<td>0.447 ± 0.045</td>
<td>0.444 ± 0.057</td>
<td>0.457 ± 0.040</td>
<td>0.78</td>
<td></td>
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<tr>
<td><strong>Arginine, μmol/L</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>106.2 ± 8.2</td>
<td>106.2 ± 15.4</td>
<td>98.2 ± 13.5</td>
<td>0.41</td>
<td>0.3 (-7.9 to 8.5)</td>
<td>3.8 (-4.9 to 12.6)</td>
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</tr>
<tr>
<td>HMR 2.5</td>
<td>108.7 ± 14.9</td>
<td>108.5 ± 15.9</td>
<td>103.3 ± 15.0</td>
<td>0.17</td>
<td>5.8 (-3.0 to 14.7)</td>
<td>10.0 (1.7 to 18.3)</td>
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</tr>
<tr>
<td>HMR 10</td>
<td>97.3 ± 19.5</td>
<td>100.9 ± 19.8</td>
<td>98.7 ± 18.2</td>
<td>0.39</td>
<td>-3.2 (-11.6 to 5.2)</td>
<td>1.7 (-6.3 to 9.7)</td>
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</tr>
<tr>
<td>HMR 50</td>
<td>101.1 ± 18.1</td>
<td>97.4 ± 14.6</td>
<td>92.9 ± 14.4</td>
<td>0.09</td>
<td>-0.3 (-8.9 to 8.4)</td>
<td>5.9 (-2.5 to 14.4)</td>
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<tr>
<td>Raloxifene</td>
<td>101.0 ± 15.6</td>
<td>100.3 ± 15.9</td>
<td>99.3 ± 13.5</td>
<td>0.19</td>
<td></td>
<td></td>
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<tr>
<td><strong>SDMA, μmol/L</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>0.478 ± 0.062</td>
<td>0.504 ± 0.067</td>
<td>0.509 ± 0.067</td>
<td>0.02</td>
<td>-8.9 (-14.9 to -2.9)</td>
<td>-8.8 (-15.9 to -1.7)</td>
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<tr>
<td>HMR 2.5</td>
<td>0.517 ± 0.070</td>
<td>0.498 ± 0.065</td>
<td>0.506 ± 0.073</td>
<td>0.01</td>
<td>-6.8 (-13.0 to -0.7)</td>
<td>-11.7 (-19.7 to -3.8)</td>
<td></td>
</tr>
<tr>
<td>HMR 10</td>
<td>0.513 ± 0.068</td>
<td>0.508 ± 0.076</td>
<td>0.488 ± 0.060</td>
<td>0.01</td>
<td>-9.9 (-15.2 to -3.3)</td>
<td>-16.2 (-22.4 to -10.0)</td>
<td></td>
</tr>
<tr>
<td>HMR 50</td>
<td>0.550 ± 0.078</td>
<td>0.528 ± 0.075</td>
<td>0.502 ± 0.061</td>
<td>&lt; 0.01</td>
<td>-7.1 (-13.0 to -1.2)</td>
<td>-7.2 (-14.1 to -0.2)</td>
<td></td>
</tr>
<tr>
<td>Raloxifene</td>
<td>0.488 ± 0.044</td>
<td>0.481 ± 0.059</td>
<td>0.493 ± 0.065</td>
<td>0.01</td>
<td>-3.9 (-5.8 to 13.6)</td>
<td>3.8 (-5.1 to 12.6)</td>
<td></td>
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<tr>
<td><strong>Arginine/ADMA ratio</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>241 ± 36</td>
<td>231 ± 31</td>
<td>221 ± 28</td>
<td>0.21</td>
<td>3.9 (-5.8 to 13.6)</td>
<td>3.8 (-5.1 to 12.6)</td>
<td></td>
</tr>
<tr>
<td>HMR 2.5</td>
<td>237 ± 37</td>
<td>236 ± 41</td>
<td>223 ± 40</td>
<td>0.12</td>
<td>7.0 (-2.4 to 16.5)</td>
<td>10.9 (3.1 to 18.7)</td>
<td></td>
</tr>
<tr>
<td>HMR 10</td>
<td>216 ± 44</td>
<td>219 ± 38</td>
<td>220 ± 40</td>
<td>0.24</td>
<td>4.5 (-4.2 to 13.2)</td>
<td>8.7 (1.1 to 16.3)</td>
<td></td>
</tr>
<tr>
<td>HMR 50</td>
<td>224 ± 53</td>
<td>226 ± 40</td>
<td>217 ± 39</td>
<td>0.35</td>
<td>3.6 (-4.8 to 12.0)</td>
<td>4.1 (-3.5 to 11.7)</td>
<td></td>
</tr>
<tr>
<td>Raloxifene</td>
<td>227 ± 34</td>
<td>227 ± 39</td>
<td>219 ± 37</td>
<td>0.40</td>
<td></td>
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</tr>
</tbody>
</table>

Table 2: Concentrations and ratios are given as mean ± SD. ANCOVA, analysis of covariance; ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine; HMR 2.5, HMR 3339 2.5 mg; HMR 10, HMR 3339 10 mg; HMR 50, HMR 3339 50 mg; raloxifene, raloxifene 60 mg.

<sup>a</sup> Analysis of covariance (ANCOVA) for repeated measurements for among-group differences, with the baseline value of the variable under consideration as constant covariate, over the 12-week study period.

<sup>b</sup> ANCOVA over the 12-week period: placebo vs treatment. Other significant between-group differences were found for ADMA between the HMR 2.5, HMR 10 and raloxifene groups vs the HMR 50 group (<i>P</i> < 0.01 for all) and for arginine between the HMR 10 and HMR 50 groups (<i>P</i> < 0.05).

<sup>c</sup> Percentage changes are mean (95% CI) of the individual percentage changes from baseline compared with placebo at week 4 and week 12.

<sup>d</sup> One-way analysis of variance, cross-sectional comparison for among-group differences.
4. DISCUSSION

Moderate reductions in ADMA levels observed in the present study could be of clinical relevance.

Also supporting the conclusion that the changes seen in this study might be clinically relevant is the observation of a significant correlation between the absolute change in concentrations of ADMA and arginine. This could represent a change in NOS activity. This positive correlation suggests that lowering of ADMA leads to a reduction of NOS inhibition and hence increased NO production and concomitant arginine consumption.

There were no changes in ADMA concentrations with raloxifene 60 mg/day in this study, in accordance with the negative results of a long-term study with a dose of 150 mg/day.22

In contrast to HT, it seems that SDMA concentrations were more affected by HMR 3339 and raloxifene than were ADMA concentrations.22–24 The SDMA reductions found in all active treatment groups were statistically significant compared with placebo. It is possible, however, that this is (partly) due to the increase in SDMA concentration of approximately 8% seen in the placebo group. The observed increase in the placebo group could be time related, although this is not very likely since the study lasted only 12 weeks. It may be the case that despite there being no changes in the raloxifene group compared with baseline, raloxifene could inhibit the SDMA increase over time. At first sight the reduction in the HMR 50 group could be a regression to the mean phenomenon, since the baseline value of SDMA in this group is significantly higher than it is in the other groups. However, we adjusted for this difference by correcting for the baseline value of SDMA in the ANCOVA.

In contrast to ADMA, SDMA has no inhibitory effect on NOS. However, SDMA does interfere with NO metabolism by competing with arginine, the substrate of NOS, for cellular transport across the membrane resulting in an intracellular arginine depletion.25 In addition, both ADMA and SDMA may compete with arginine for tubular reabsorption in the kidney, thereby reducing arginine availability for NO production.28 Although the association between ADMA and cardiovascular events is well established, this association for SDMA is less pronounced.18,21,29,30 This does not, however, mean that SDMA does not play a role in the pathophysiology of CHD. It is possible that the reduction in SDMA by HMR 3339 has a beneficial effect on CHD risk.

SDMA is mainly cleared by renal filtration, whereas only a small part of ADMA is cleared from the circulation through this pathway.31 Approximately 80% of ADMA is metabolized by the widely expressed enzyme dimethylarginine dimethylaminohydrolase.32 Although it has been speculated that HT would have an effect on dimethylarginine dimethylaminohydrolase activity, because this would influence ADMA concentrations more than SDMA concentrations, it is possible that SERMs, particularly HMR 3339, increase glomerular filtration rate, resulting in larger changes in SDMA concentrations than in ADMA concentrations.24

A major strength of this study is the randomized, placebo-controlled, double-blind design. Nevertheless, this study also has limitations. Because study groups were relatively small, minor treatment effects may have been missed. For the effects in ADMA concentrations, the observed power was 83%; however, for the changes in arginine, SDMA, and the arginine/ADMA ratio, the observed power was 48%, 77%, and 22%, respectively. Conclusions from non-significant changes in these last three observations are therefore difficult to draw.
In addition, the treatment period of our study was 12 weeks. Especially for the low-dose groups, longer treatment duration may be necessary to reach a maximal effect. Women with moderate to severe climacteric complaints were excluded from this study. Therefore, caution should be used when extrapolating the results of this study to the entire group of postmenopausal women, including the women with moderate to severe vasomotor symptoms.

5. Conclusion

In summary, after 12 weeks HMR 3339 50 mg reduced ADMA concentrations, whereas SDMA was reduced in all treatment groups compared with placebo. The reduction of the NOS inhibitor ADMA by HMR 3339 is similar to the effect of estrogen therapy and may possibly lead to increased NO production, which is generally considered to act as a brake on atherogenesis. The clinical implication of these effects for CHD risk is unclear. Conclusive evidence about the effect of ADMA and SDMA changes needs to come from long-term, randomized, placebo-controlled clinical trials investigating the relevance of changes in dimethylarginine concentrations for CHD events.

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References


(32) Achan V, Broadhead M, Malaki M, et al. Asymmetric dimethylarginine causes hypertension and cardiac dysfunction in humans and is actively
1. Introduction

The aim of this thesis was to assess the effects of menopause, of post-menopausal hormone therapy (HT) and of alternatives to HT, on blood concentrations of the independent arterial disease risk marker, asymmetric dimethylarginine (ADMA) in apparently healthy women. This thesis addressed the following questions:

- Does menopause, either physiological or surgical, influence ADMA concentrations?
- Does oral 17β-oestradiol therapy influence ADMA concentrations in post-menopausal women?
- Does adding a progestogen change the effect of oral 17β-oestradiol therapy on ADMA concentrations?
- Does the effect of HT on ADMA concentrations depend on the route of administration?
- And finally, are ADMA concentrations influenced by alternatives for HT, such as plant-derived substances or selective oestrogen receptor modulators (SERMs)?

In the following sections the answers to these questions are discussed in the light of the results of the studies described in this thesis and relevant data from the literature. In addition, we shortly hypothesise on mechanisms that may be involved in the effects of HT on ADMA concentrations and on the clinical implications of these effects.

2. ADMA and menopause

Women over the age of 50 years have been shown to have a higher mean plasma ADMA concentration compared with women under the age of 50 years. While increasing age is associated with increasing ADMA concentrations this association was not found in men over the age of 50 years compared with younger men. As the physiological menopause occurs at a mean age of approximately 51 years, this observation suggests an influence of menopause on ADMA concentrations in women. Neither the distribution of pre- and postmenopausal women in the population under the age of 50 years and in the population over the age of 50 years nor the distribution of women using HT in these two groups was known in the previously
The lack of this information weakens the hypothesised association between menopause and ADMA concentrations based on this observational study.

In our study, postmenopausal women had higher ADMA blood concentrations compared with age-matched premenopausal women (Chapter 2), supporting the idea that, following the menopausal transition, ADMA concentrations increase in women on top of the age-related-increase in ADMA concentrations. Interestingly, the mean 9.5% higher ADMA concentration found in early postmenopausal women compared with premenopausal women is of the same magnitude as the decrease of approximately 8% in ADMA concentrations found after oral oestrogen therapy (Chapters 3, 4 and 5).

Our longitudinal study with a four-year follow-up of women going through menopause could not confirm the observed menopause-related ADMA increase (Chapter 2). In this longitudinal study serum hormone concentrations (oestradiol, follicle-stimulating hormone (FSH), inhibin A and B) changed according to the decline in ovarian function after the menopause. Apart from homocysteine, none of the other arterial disease risk markers investigated (lipids, weight, body mass index, leptin and C-reactive protein) showed changes over time in these women. Possibly, although the hormone levels changed rapidly during the menopausal transition, a longer observation period is needed for the effects of these hormonal changes on the arterial disease markers to become detectable and maybe the two-year follow-up after the moment of menopause in this study was too short in this respect.

In the first two years of the study, that is the two years directly before the moment of menopause (premenopausal), many women had low premenopausal oestradiol and high FSH concentrations. It is possible that the influence of the oestradiol reduction and FSH increase on ADMA concentrations in these women had already partly occurred before study participation. Hormone fluctuations in the period before the menopause have been observed earlier. Possibly a wider time window around the menopause is needed to find menopause-related effects on arterial disease markers in general and on ADMA concentrations in particular.

In the group of women with a prophylactic bilateral salpingo-oophorectomy, the hormone profile changed from a premenopausal to a postmenopausal profile. The mean ADMA concentration increased approximately 5% (non-significantly) after the operation (Chapter 2).

In conclusion, ADMA concentrations increased in women going through the menopause on top of the age-related ADMA increase and this increase may contribute to the deterioration of the health of the vascular system, resulting in a higher arterial disease risk.

3. ADMA and oestrogen therapy

Both oral 17β-oestradiol and oral conjugated equine oestrogens (CEE) reduced ADMA plasma concentrations to a similar extent (approximately 8%) (Chapters 3, 4 and 5). This indicates that the moderate ADMA lowering effect of oestrogens is not a spurious finding but a real phenomenon. Although the absolute effect may seem small, it is noteworthy that the biological variation of plasma ADMA concentrations is very low, with an interindividual coefficient of variation of approximately 10%. Even a slight increase in ADMA concentrations (above 0.62 μmol/L)
was demonstrated to be associated with a strongly elevated risk for acute coronary events. Therefore, in our opinion the moderate reductions in ADMA levels as observed with 17β-oestradiol and CEE could well be of clinical relevance.

After four weeks of treatment with oral 17β-oestradiol, a reduction of approximately 6% of ADMA concentrations was observed (Chapter 4). After 12 weeks and one year of 17β-oestradiol administration, ADMA concentrations were reduced by 8% (Chapters 3 and 4). The mean ADMA concentration was reduced by 7% after six months and after one year and 10% after two years of oral CEE treatment. These observations suggest that short-term effects are sustained for at least two years. Both 1 mg and 2 mg 17β-oestradiol administered orally reduced ADMA concentrations to a similar extent (Chapters 3 and 4) suggesting no relevant dose-dependent effect of 17β-oestradiol on ADMA concentrations. A randomised, placebo-controlled trial comparing the effects of various dosages of 17β-oestradiol on ADMA concentrations is necessary to see whether this is also true for low and ultra low dosages of 17β-oestradiol.

Both oral 17β-oestradiol and CEE reduced ADMA concentrations to a similar extent, suggesting no difference between these types of exogenous oestrogens. Two weeks after implants of 100 mg ethynyloestradiol were administered in the subcutaneous fat of the abdominal wall, the mean ADMA concentration decreased approximately 19% in postmenopausal women. This could be interpreted such that ethynyloestradiol has a stronger reducing effect of ADMA concentrations than 17β-oestradiol and CEE have. However, most probably another explanation such as the use of a higher dosage of ethynyloestradiol per day could explain the difference in magnitude of the reduction. Furthermore, it was not a placebo-controlled study and moreover, menopause was not defined, which makes it difficult to compare the study populations of the different studies. Only a randomised, placebo-controlled trial comparing these three different oestrogens head-to-head administered via the same route with comparable dosages can demonstrate a real difference, if any. A problem inherent in such a study design is the definition of comparable dosages of the three regimens with similar clinical and metabolic activity.

What mechanism is thought to explain the oestrogen-lowering effect on ADMA blood concentrations? Symmetric dimethylarginine (SDMA) is mainly cleared by renal excretion, whereas only a small part of ADMA is cleared from the circulation by this pathway. Since ADMA concentrations are affected to a larger degree by oestrogens than SDMA concentrations, an increase in glomerular filtration rate as a cause for the ADMA-lowering effect of HT is unlikely, because this would affect SDMA concentrations to a larger extent than ADMA concentrations.

Approximately 80% of ADMA is metabolised by the widely expressed enzyme dimethylarginine dimethylaminohydrolase (DDAH). DDAH is very sensitive to oxidative stress since the active site of the enzyme contains a critical sulphhydryl group that is required for its catalytic activity. Consequently, pathological stimuli that induce oxidative stress, such as hyperhomocysteinemia and oxidised low-density lipoprotein cholesterol, may reduce DDAH activity and lead to an accumulation of ADMA. Conversely, compounds with anti-oxidant properties, possibly including oestrogens, may protect DDAH from inactivation by oxidants, leading to an increase in ADMA metabolism. Exposure of human and murine endothelial cells to 17β-oestradiol resulted in an increased activity of DDAH and was accompanied by a reduced release of ADMA.
Both ADMA and SDMA are formed by methylation of arginine residues in proteins by protein arginine methyltransferases and free ADMA and SDMA are released upon proteolysis of these methylated proteins. Oxidative stress may also lead to accelerated proteasomal degradation of proteins. It is thus conceivable that reduction of oxidative stress by HT, by reducing proteolysis of methylated proteins, leads to a diminished generation of both ADMA and SDMA. The combination of a decreased proteolysis and enhanced DDAH activity would explain the decrease of both ADMA and SDMA, the reduction of ADMA being more pronounced.

In summary, different types of oestrogen therapies reduced ADMA concentration in postmenopausal women. Knowledge about the mechanism through which oestrogens and probably other oestrogen-receptor ligands could influence plasma ADMA concentrations is far from complete. Future research should focus on disclosing the mechanism(s) underlying the oestrogenic effects on ADMA concentrations. ADMA and combined oestrogen plus progestogen therapy

A progestogen is added to oestrogen therapy in women with a uterus to prevent the development of neoplasia of the endometrium. Progestogens used in preparations can be either derivates from progesterone or from testosterone. The four progestogens investigated in this thesis are dydrogesterone and trimegestone, which are derivates of progesterone, and gestodene and norethisterone, which are testosterone derivates. Dydrogesterone and gestodene seemed to have no effects on the 17β-oestradiol-induced ADMA reduction (Chapters 3 and 4), whereas trimegestone augmented the effects of 17β-oestradiol on the ADMA plasma concentration (Chapter 3).

The effect of an oral combination of 17β-oestradiol and norethisterone acetate on ADMA concentrations was investigated as well (Chapter 5). Norethisterone is a 19-nortestosterone derivative with a partial androgenic activity, which can reverse oestrogen-induced effects independent of its route of administration. The oral combination reduced ADMA concentrations with 7%. This decrease was similar to the decrease found with 17β-oestradiol alone and 17β-oestradiol combined with dydrogesterone (Chapter 3) and gestodene (Chapter 4). Both these progestogens did not modify the ADMA-reducing effect of 17β-oestradiol. Possibly, norethisterone acetate also did not change the ADMA-reducing effect of oestrogen as well. A shortcoming in this study is the lack of a 17β-oestradiol alone group.

All four progestogens have a strong affinity for the progesterone receptor, but they probably differ in their trans-activation effects. This might explain some of the observed differences in the effects of the four progestogens on the 17β-oestradiol-induced changes in ADMA concentrations.

If reducing ADMA concentrations was to be a target in the treatment and prevention of arterial disease in women with severe menopausal symptoms requiring HT, the combination of 17β-oestradiol with trimegestone would have the most potent reducing effect on ADMA concentrations.

4. ADMA and route of HT administration

Transdermally administered 17β-oestradiol reduced ADMA plasma concentrations less than oral 17β-oestradiol (Chapter 4), and intranasal 17β-oestradiol combined with norethisterone seemed to have no effect on ADMA plasma concentrations compared with orally administered 17β-oestradiol combined with norethisterone acetate (Chapter 5). An explanation for these differences may be found in the role
of the liver in ADMA metabolism or in the difference in pharmacokinetics of the different administration forms.

It has been shown in both rats and humans that the liver plays an important role in the elimination of ADMA, probably through the degradation of ADMA by DDAH which is extensively present in the liver.\textsuperscript{23,24} This may provide an explanation for the observed difference between the transdermal and intranasal routes on the one hand versus the oral route of HT administration on the other. Unlike oral oestrogens, transdermally and intranasally administered oestrogens directly enter the systemic circulation without first passing through the liver. The larger effect of oral therapy compared to transdermal and intranasal therapy thus possibly reflects the crucial role of the liver in the elimination of ADMA and is in line with stimulation of DDAH activity by oestrogens.\textsuperscript{10}

After intranasal administration, oestradiol is rapidly absorbed and induces a very steep and short-lived peak in plasma levels, whereas oral administration causes a prolonged oestrogen exposure.\textsuperscript{25,26} Perhaps, a prolonged exposure to a minimum level of oestrogens is needed for an adequate reduction of ADMA concentrations, and the transient oestradiol peak after intranasal administration is not effective in this respect.\textsuperscript{26}

While transdermal 17$\beta$-oestradiol significantly reduced ADMA concentrations (compared to baseline and placebo), the intranasal route had no effect on ADMA concentration (compared to baseline). The intranasal route was not compared with a placebo group and therefore it can only be concluded that the intranasal route showed no effects on ADMA concentrations compared with the oral group.

A difference between the transdermal administration (Chapter 4) and the intranasal administration (Chapter 5) is the addition of norethisterone to the intranasal route. However, as was argued earlier, when comparing the effect in the oral group with the results in 17$\beta$-oestradiol-alone groups from our other studies (Chapters 3 and 4) it seemed that oral norethisterone acetate did not modify the oral 17$\beta$-oestradiol-induced ADMA reduction. This is probably not different for the intranasal administration of the combination of 17$\beta$-oestradiol and norethisterone.

In conclusion, oral HT reduces ADMA concentrations more than transdermal and intranasal administered HT. Only a randomised comparative study using equivalent dosages of 17$\beta$-oestradiol for the transdermal and the intranasal routes can demonstrate the superiority of one of these administration routes over the other in reducing ADMA concentrations in women. Besides the requirement of comparable dosages, both treatments should contain either 17$\beta$-oestradiol alone or 17$\beta$-oestradiol combined with the same progestogen.

5. ADMA and alternatives for HT

Since long-term HT has been related with health risks\textsuperscript{27–30} it is necessary to explore alternative treatment options with a benefit/risk profile that is more acceptable for the treatment of symptomatic menopausal women. These may be found among plant-derived substances or SERMs. In this thesis the effects of a supplement containing both soy isoflavones and Actaea racemosa Linnaeus (Actaea racemosa L.) and the SERMs HMR 3339 and raloxifene on ADMA concentrations were studied.

Eight weeks of only soy isoflavones\textsuperscript{31} as well as 12 weeks of a combination of soy isoflavones and Actaea racemosa L. (Chapter 6) did not influence ADMA plasma
concentrations in healthy postmenopausal women. Daily 60 mg (Chapter 7) as well as 150 mg raloxifene \(^7\) did not change ADMA plasma concentrations, whereas HMR 3339 showed a dose-dependent reduction in ADMA plasma concentrations (Chapter 8). HMR 3339 had a dose-dependent reducing effect on SDMA plasma concentrations as well. This reduction of SDMA concentrations by HMR 3339 was more pronounced than the reduction of ADMA concentrations, whereas HT had less effect on SDMA than on ADMA concentrations. In contrast to ADMA, SDMA has no inhibitory effect on nitric oxide synthase. However, by competing with arginine for cellular uptake, SDMA may interfere with nitric oxide synthesis by reducing arginine availability.\(^{32,33}\) While the association between ADMA and arterial disease is well established, this association for SDMA is less pronounced.\(^{34–37}\) This does, however, not mean that SDMA does not play a role in the pathophysiology of arterial disease. It is possible that the reduction in SDMA by HMR 3339 has a beneficial effect on arterial disease risk.

In conclusion, soy isoflavones alone or in combination with *Actaea racemosa* L. did not modify ADMA concentrations in healthy postmenopausal women. The SERM HMR 3339 did reduce both ADMA and SDMA concentrations and this could represent a favourable effect on arterial health in postmenopausal women.

### 6. Clinical relevance

ADMA blood concentrations are associated with present and future arterial disease (Table 1, Chapter 1).\(^9,35,37–41\) Lu et al. observed lower arterial disease risk in patients with reduced ADMA concentrations after percutaneous coronary intervention, whereas in patients in whom ADMA concentrations remained high, arterial disease risk was significantly higher than in the first group.\(^38\) It is difficult to tell whether the low ADMA concentration after the intervention caused a reduction in arterial disease risk or whether the decreased ADMA concentrations were the result of reduced arterial disease risk. Therefore, it still remains unclear whether or not reducing ADMA concentrations by therapeutic agents or other measures will lead to a reduction in severity of prevalent arterial disease and/or reduce the risk for future arterial disease.

Assuming that a reduction of ADMA concentrations improves vascular health, another interesting question arises: Are oestrogens a viable option for ADMA reduction? As follows from Table 4 in Chapter 1 and the results of the studies described in this thesis, in comparison with oestrogen therapy a larger reduction in ADMA concentrations can be achieved by treatment with other therapeutic agents like angiotensin converting enzyme inhibitors, angiotensin receptor blockers, and possibly hypoglycaemic agents. As far as we can conclude now, only oestrogens combined with trimegestone could compete with these treatments regarding the magnitude of ADMA reduction (Chapter 3).

Angiotensin converting enzyme inhibitors, angiotensin receptor blockers and hypoglycaemic agents were tested in populations with high arterial disease risk. The populations in our studies were healthy postmenopausal women with no arterial disease in their history and not using treatment for reducing arterial disease risk. It is possible that oestrogens have a larger effect on (pathologic) high ADMA concentrations in a population at higher risk for arterial disease. The mechanism behind the ADMA-reducing effect of oestrogens and the role of oestrogens in vascular health in women should therefore be explored further in future research.
Besides the fact that there are other treatments with larger effects on ADMA concentrations, oestrogens alone or combined with a progestogen also carry health risks, such as venous thromboembolism and stroke. Furthermore, HT has negative effects on some other arterial disease risk markers, including markers reflecting coagulation and inflammation. While there is evidence that HT should not be used as an arterial disease prevention strategy in late postmenopausal women, the role of HT as an arterial disease prevention strategy in early postmenopausal women remains unclear. A large randomised placebo-controlled trial with hard clinical arterial disease endpoints in healthy early postmenopausal women could be conclusive here. However, a very large number of women need to be followed-up for a long period of time. As many of these women suffer from menopausal complaints, a placebo control group will not be feasible. Furthermore, such a study needs astronomical financial funds, which are hard to find. Thus far, HT is recommended only as a treatment option for women suffering from perimenopausal complaints and not for arterial disease prevention.

In summary, ADMA is increased after the menopause in apparently healthy women. Oral oestrogen therapy alone or combined with a progestogen reduced ADMA concentrations and oral HT had a larger effect on ADMA concentrations than transdermally or intranasally administered HT. Neither soy isoflavones combined with Actaea racemosa L., nor the SERM raloxifene, modified ADMA concentrations, whereas the SERM HMR 3339 had a dose-dependent reducing effect on both ADMA and SDMA. The clinical relevance of the effects of menopause and menopausal therapies on ADMA concentrations in middle-aged women is not yet known. It could be speculated that the increase in ADMA concentrations after the menopause contributes to the deterioration of cardiovascular health in postmenopausal women, and HT, through its ADMA-lowering effect, partly prevents this deterioration. However, more well-designed clinical studies are needed to deliver the evidence for these speculations.

References


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Summary

This thesis evaluates the relationship between endogenous or exogenous oestrogen or oestrogen-related substances and asymmetric dimethylarginine (ADMA), an endogenous nitric oxide synthase (NOS) inhibitor and arterial disease risk marker, in middle-aged women. The effect of endogenous oestrogens was investigated in a longitudinal study of women going through the menopausal transition, in a case-control study in which postmenopausal women were compared with age-matched premenopausal women and in a study of women with a surgical menopause. In five randomised clinical trials, the effects of oral and non-oral oestrogen therapies as well as oral oestrogen therapy combined with a progestogen and oestrogen-related substances on ADMA concentrations were investigated. Concentrations of ADMA, arginine and symmetric dimethylarginine (SDMA) were measured by high-performance liquid chromatography in all studies described in this thesis.

Background

Arterial disease is the main cause of death in the industrialised countries. Premenopausal women have lower arterial disease incidence than men of the same age. After the menopause, the risk for arterial disease increases rapidly in women. Possibly, a strong reduction in oestrogen production after the menopause plays an important role in this acceleration of the arterial disease risk in women. Observational studies indicated that the use of oestrogen therapy alone or combined with a progestogen (both will further be referred to as hormone therapy (HT)) could protect postmenopausal women from this increase in arterial disease risk. However, large randomised placebo-controlled trials reported no cardiovascular benefit of long-term oral HT use, and possibly even an early increase of arterial disease risk in late postmenopausal women. The effects of HT in early postmenopausal women as well as the effects of non-orally administered HT on arterial disease risk in postmenopausal women are still unclear.

Studies with large numbers of women are needed to study the influence of endogenous or exogenous oestrogens on hard clinical arterial disease endpoints such as myocardial infarction and stroke. An interesting alternative could be to investigate the influence of oestrogens on arterial disease risk markers instead. Many of these risk markers are related to different processes involved in the pathogenesis of arterial disease, such as coagulation, lipid metabolism, endothelial function, inflammation and oxidation.

Nitric oxide (NO) is a potent vasodilator produced by the endothelium. A reduced NO availability has been suggested to play a role in the development of
arterial disease. ADMA, an endogenously produced methylated form of arginine, inhibits the enzyme NOS, resulting in low NO concentrations. High blood levels of ADMA have been associated with increased risk of cardiovascular events and mortality risk in specific patients populations. Male participants dominated most of these study populations.

From the available literature, a link between estrogens and ADMA can be inferred. However, the influence of menopause on ADMA concentrations in women is not clear and reports on the effect of HT on ADMA concentrations in postmenopausal women are scarce. Therefore, this thesis addressed the following question: Do menopause, HT and alternatives for HT modify ADMA concentrations in middle-aged women?

**ADMA and menopause**

The changes in ADMA concentrations resulting from a physiological and a surgical menopause are evaluated in Chapter 2. In a longitudinal study, women were examined annually from two years before until two years after physiological menopause, and in a case-control study, postmenopausal women were compared with age-matched premenopausal women. Surgical menopause effects were investigated in women undergoing a prophylactic bilateral salpingo-oophorectomy. The following parameters were measured: serum concentrations of oestradiol, follicle-stimulating hormone (FSH), inhibin A, inhibin B, ADMA, lipids, leptin, homocysteine, C-reactive protein (CRP) and coenzyme Q10. Length and weight were measured as well, and the body mass index was calculated.

After the physiological and surgical menopause, serum oestradiol and inhibin A and B decreased, whereas FSH increased. Serum ADMA, total and low-density lipoprotein (LDL-)cholesterol and leptin concentrations were significantly higher in postmenopausal women compared to premenopausal women and serum homocysteine increased during the menopausal transition. Furthermore, total and LDL-cholesterol increased after the surgical menopause as well. None of the other parameters was influenced statistically significantly by the menopausal transition.

In conclusion, the arterial disease risk profile is affected unfavourably by menopause. Changes in most arterial disease risk markers were small, despite substantial changes in the hormonal parameters studied.

**ADMA and oral oestrogen-only or combined oestrogen-progestogen therapy**

In Chapter 3 the effects of short-term oral HT on ADMA, arginine and SDMA were investigated in a prospective, randomised, placebo-controlled 12-week study. Healthy postmenopausal women received daily placebo or oral 17β-oestradiol, either unopposed or sequentially combined with dydrogesterone or trimegestone. Fasting plasma concentrations of ADMA, arginine and SDMA were measured at baseline and after four and twelve weeks.

ADMA concentrations reduced in all active treatment groups. Compared to baseline and placebo, the largest reduction in ADMA levels was observed after 17β-oestradiol combined with trimegestone (-18.7% and -21.1% at four and twelve weeks, respectively). At four and twelve weeks, this combination significantly reduced arginine concentrations as well (-30.9% and -36.3%, respectively). SDMA
concentrations were significantly lower after 17β-oestradiol combined with dydrogesterone after twelve weeks (-11.6%).

In conclusion, oral 17β-oestradiol, either alone or combined with dydrogesterone or trimegestone, reduced plasma levels of the NOS inhibitor ADMA. The largest reduction was seen after 17β-oestradiol combined with trimegestone. Whether the reduction of the NOS substrate arginine in the group receiving 17β-oestradiol combined with trimegestone counteracts the potentially beneficial effect of ADMA reduction or only reflects increased NO production remains to be investigated.

In Chapter 4, the effects of oral unopposed 17β-oestradiol versus 17β-oestradiol continuously combined with gestodene were investigated in a placebo-controlled, double-blind study. The study duration was 13 (28-day) cycles and fasting plasma concentrations of ADMA, arginine and SDMA were measured at baseline and in treatment cycles four and 13.

Unopposed oral 17β-oestradiol reduced ADMA concentrations with 7.7% and combined therapy reduced ADMA concentrations by 7.5% compared with placebo after 13 cycles. Both treatment regimens significantly reduced arginine concentrations compared to placebo as well. Only unopposed 17β-oestradiol treatment significantly reduced SDMA concentrations. In summary, adding gestodene to oral 17β-oestradiol did not modify the reduction in ADMA concentrations seen with 17β-oestradiol alone therapy.

The effect of an oral combination of 17β-oestradiol and norethisterone acetate on ADMA concentrations was investigated as well (Chapter 5). The oral combination reduced ADMA concentrations with 7%. This decrease was similar to the decrease found with 17β-oestradiol alone (Chapter 3 and 4) and 17β-oestradiol combined with dydrogesterone (Chapter 3) and gestodene (Chapter 4). Both these progestogens did not modify the ADMA-reducing effect of 17β-oestradiol. Possibly, norethisterone acetate also does not change the ADMA-reducing effect of oestrogen as well.

ADMA and route of HT administration

In the same study as described in Chapter 4, the effects of transdermal versus oral 17β-oestradiol on ADMA concentrations were also investigated. After oral 17β-oestradiol administration a significantly larger reduction in ADMA concentration was observed than after transdermal administration. Oral, but not transdermal treatment, significantly reduced arginine and SDMA concentrations compared to placebo.

The difference in influence of intranasal versus oral 17β-oestradiol combined with norethisterone (acetate) on ADMA concentrations in postmenopausal women was investigated in a study reported in Chapter 5. In a randomised, double-blind, comparative study, healthy postmenopausal women daily received intranasally or orally administered 17β-oestradiol combined with norethisterone (acetate), in comparable dosages. At baseline, week twelve and 52, fasting plasma concentrations of ADMA, arginine and SDMA were measured.

ADMA concentrations reduced with 7.4% after oral administration, while after intranasal administration no effect (0.8%) was observed after 52 weeks. In both groups, arginine decreased transiently by approximately 6% compared to baseline at week twelve. Only oral administration reduced SDMA concentrations. Therefore,
the two studies of Chapters 4 and 5 provide evidence that both transdermally and intranasally administered HT are not as effective in reducing ADMA and SDMA concentrations as orally administered HT.

**ADMA and alternatives for HT**

Chapter 6 discusses the effects of a supplement containing soy isoflavones and *Actaea racemosa L.* on several arterial disease risk markers in menopausal women. In a randomised, placebo-controlled, double-blind study, menopausal women received daily either placebo or a supplement containing soy isoflavones and *Actaea racemosa L.* for twelve weeks. Fasting concentrations of ADMA, lipids and CRP were measured at baseline and week twelve.

In the supplement group, total cholesterol and LDL-cholesterol showed a small reduction at week twelve (both -0.2 mmol/l). Concentrations of ADMA, arginine, SDMA, triglycerides, lipoprotein(a) and CRP did not change significantly. After the 12-week study period, none of the parameters investigated revealed significant between-group differences.

Therefore, twelve weeks of administration of a supplement containing soy isoflavones and *Actaea racemosa L.* had little or no influence on the arterial disease risk markers studied. This supplement probably has neither protective nor adverse effects on the cardiovascular system, however, large long-term studies are needed to confirm this.

In addition, in Chapter 7 in the same women described in Chapter 6 we investigated the influence of the supplement intervention on menopausal symptoms as well. The modified Kupperman Index, the Greene Climacteric Scale, a visual analogue scale designed to measure quality of life and the daily number and severity of hot flushes, were evaluated at screening and at weeks six and twelve.

At weeks six and twelve, all scores in both groups had improved compared with baseline, though the overall difference in scores between the groups was not statistically significant. Therefore, the supplement containing soy isoflavones and *Actaea racemosa L.* had no additional effect compared with placebo on menopausal symptoms in women experiencing at least five vasomotor symptoms per day.

The short-term effects of three different doses of the SERM HMR 3339 in comparison with placebo and raloxifene on ADMA, arginine and SDMA concentrations are described in Chapter 8. In a randomised, placebo-controlled, double-blind, dose-ranging study, healthy postmenopausal women received daily either placebo, HMR 3339 2.5 mg, HMR 3339 10 mg, HMR 3339 50 mg, or raloxifene 60 mg for twelve weeks. Fasting plasma concentrations of ADMA, arginine and SDMA were measured at baseline and after four and twelve weeks.

HMR 3339 induced a dose-dependent reduction of ADMA and SDMA concentrations, with the largest effects in the HMR 3339 50 mg group compared to baseline and placebo (at twelve weeks: -7.0%, for ADMA and -16.2%, for SDMA). Twelve weeks of raloxifene 60 mg significantly reduced SDMA but not ADMA concentrations. Arginine concentrations were not changed by any treatment. These results suggest that HMR 3339 may have a potentially beneficial effect on the cardiovascular system by reducing the NOS inhibitor ADMA in postmenopausal women.
Conclusion

The results of the present thesis are discussed in Chapter 9. In this same chapter, briefly is speculated upon the implications for the clinical practice of these results and some suggestions are made for future research. Both endogenous and exogenous oestrogens as well as the SERM HMR 3339 reduced ADMA concentrations in healthy middle-aged women. Adding the progestogen trimegestone to oral oestrogen therapy augmented the oestrogen-induced ADMA reduction, whereas adding dydrogesterone or gestodene to oral oestrogen therapy did not modify this reduction in ADMA concentrations. In addition, norethisterone acetate did not seem to modify the oestrogen-induced ADMA reduction. Neither transdermally nor intranasally administered HT was as effective in reducing ADMA and SDMA concentrations as orally administered HT. Also, neither soy isoflavones combined with Actaea racemosa L., nor the SERM raloxifene modified ADMA concentrations.

There is a clear relation between endogenous oestrogen concentrations as well as oestrogen therapy and ADMA concentrations. Higher oestrogen concentrations coincide with lower ADMA concentrations. The clinical implications of this relation between oestrogens or oestrogen-related substances and ADMA concentrations remain unclear and the mechanisms underlying the oestrogen-induced ADMA reductions are not fully understood. Future research should focus on these two aspects of the relationship between oestrogens and ADMA in women.
Nederlandse samenvatting

In dit proefschrift wordt de relatie tussen endogene oestrogenen of exogene oestrogenen of oestrogeen verwante stoffen en asymmetric dimethylarginine (ADMA), een endogene stikstofoxide synthase (NOS) remmer en een hart- en vaatziekten (HVZ) risicomarker onderzocht in vrouwen van middelbare leeftijd. Het effect van endogene oestrogenen wordt onderzocht in drie verschillende studies, een longitudinale studie waarin vrouwen gevolgd worden van pre- naar postmenopauзаal, een case-controle studie waarin postmenopauzale vrouwen worden vergeleken met premenopauzale vrouwen van gelijke leeftijd en een studie van vrouwen met een chirurgische menopauze. In vijf gerandomiseerde klinische studies worden de effecten van oraal en niet-oraal toegediende oestrogeen therapieën en orale oestrogeen therapie gecombineerd met een progestageen en oestrogeen verwante stoffen op ADMA concentraties onderzocht. ADMA, arginine en symmetrisch dimethylarginine (SDMA) concentraties zijn in alle studies van dit proefschrift gemeten door middel van high-performance liquid chromatography.

Achtergrond

HVZ vormen de belangrijkste doodsoorzaak in de westere wereld. Premenopauzale vrouwen hebben een lager risico op HVZ dan mannen van dezelfde leeftijd. Na de menopauze stijgt de kans op HVZ snel bij vrouwen. De daling in oestrogeen concentraties in het bloed na de menopauze speelt mogelijk een belangrijke rol in deze versnelde toename van het HVZ risico. Observationele studies suggererden dat oestrogeen therapie alleen of gecombineerd met een progestageen (naar beide wordt verder verwezen met hormoontherapie (HT)) postmenopauzale vrouwen zouden kunnen beschermen tegen de versnelde toename van het risico van HVZ na de menopauze. Grote gerandomiseerde placebo-gecontroleerde studies lieten echter geen preventief (en mogelijk zelfs een vroeg optredend nadelig) effect op HVZ zien bij langdurig gebruik van orale HT door vrouwen die al langere tijd postmenopauzaal waren. De effecten van HT op het HVZ risico bij vrouwen in de overgang die nog maar kort postmenopauzaal zijn is echter niet duidelijk. Ook het effect van niet-oraal toedieningsvormen van HT op het risico van HVZ bij postmenopauzale vrouwen is nog niet opgehelderd.

Grootschalige studies zijn nodig om de invloed van endogene en exogene oestrogenen op harde klinische eindpunten van HVZ zoals myocardinfarct en beroerte te onderzoeken. Een interessant alternatief wordt gevonden in de bestudering van de invloed van oestrogenen op risicomarkers voor HVZ. Vele van deze risicomarkers zijn gerelateerd aan een of meer processen die geassocieerd worden met HVZ,
zoals stolling, vetmetabolisme, endotheelfunctie, ontstekingreacties en oxidatieve processen.

Stikstofoxide (NO) is een stof die geproduceerd wordt door het endotheel en heeft een sterk vaatverwijdend effect. Er wordt verondersteld dat lage NO concentraties in het bloed een belangrijke rol spelen in het ontstaan van HVZ. ADMA is een endogeen geproduceerde gemethyleerde vorm van arginine, die het enzym NOS remt, wat resulteert in lagere NO concentraties. Hoge ADMA concentraties zijn geassocieerd met een verhoogd HVZ risico en een verhoogde mortaliteit in diverse patiëntenpopulaties. Deze populaties bestonden echter voornamelijk uit mannelijke participants.

Studie van de wetenschappelijke literatuur suggereert een mogelijk verband tussen oestrogenen en ADMA. Er zijn echter tot nu toe geen studies gepubliceerd die het effect van menopauze op ADMA concentraties in vrouwen hebben onderzocht en het effect van oestrogeen therapie op ADMA concentraties in vrouwen is zelden beschreven. Daarom zal er in dit proefschrift gepoogd worden een antwoord te geven op de volgende vraag: kunnen menopauze, HT en alternatieven voor HT ADMA concentraties beïnvloeden in vrouwen van middelbare leeftijd?

**ADMA en menopauze**


Na fysiologische en chirurgische menopauze daalden de serum concentraties van oestradiol en inhibine A en B terwijl de FSH concentratie steeg. Serum ADMA, totaal en low-density lipoproteïne (LDL-)cholesterol en leptine concentraties waren significant hoger in postmenopauzale vrouwen dan in premenopauzale vrouwen en serum homocysteïne steeg tijdens de overgangsperiode. Verder stegen het totaal en LDL-cholesterol ook na de chirurgische menopauze. Geen van de andere risicomarkers werd statistisch significant beïnvloed door de menopauze.

Uit deze bevindingen kan geconcludeerd worden dat het HVZ risicoprofiel ongunstig beïnvloed wordt door de menopauze. De veranderingen in de meeste risicomarkers waren echter klein, terwijl er substantiële veranderingen in de hormoonspiegels waarneembaar waren.

**ADMA en oraal oestrogeen al dan niet gecombineerd met een progestageen**

In Hoofdstuk 3 worden de effecten van korte termijn behandeling met orale HT op ADMA, arginine en SDMA concentraties beschreven van een prospectief, gerandomiseerde, placebo-gecontroleerde, twaalf weken durende studie. Gezonde
postmenopauzale vrouwen kregen dagelijks óf placebo, óf oraal 17β-oestradiol zonder een progestageen, óf oraal 17β-oestradiol sequentieel gecombineerd met dydrogesteron of trimegeston. ADMA, arginine en SDMA concentraties werden gemeten in nuchter afgenomen plasma op baseline en na vier en twaalf weken.

ADMA concentraties daalden in alle groepen behalve in de placebo groep. De grootste daling in ADMA concentraties werd gevonden na behandeling met 17β-oestradiol gecombineerd met trimegeston (-18,7% na vier weken en -21,1% na twaalf weken) in vergelijking met baseline en de placebo behandeling. Dezelfde behandeling verlaagde ook de arginine concentraties na vier en twaalf weken (30,9% en -36,3%, respectievelijk). Na twaalf weken daalden de SDMA concentraties in de groep die behandeld werd met 17β-oestradiol gecombineerd met dydrogesteron (-11,6%).

Dus oraal 17β-oestradiol, alleen of gecombineerd met dydrogesteron of trimegeston, verlaagde de plasma concentraties van de NOS remmer ADMA. De grootste daling werd gezien na de combinatie van 17β-oestradiol met trimegeston. Of de door de combinatie van 17β-oestradiol met trimegeston veroorzaakte daling in arginine, het substraat van NOS, een mogelijk gunstig effect op ADMA concentraties tegnerwerkt of dat het alleen maar een stijging van de NO productie weerspiegelt, moet verder worden onderzocht.

In Hoofdstuk 4 wordt het effect van oraal 17β-oestradiol alleen vergeleken met een combinatie van 17β-oestradiol met gestodeen in een placebo-gecontroleerde, dubbel-blinde studie. De studie duurde 13 cycli (van 28-dagen). Nuchtere plasma concentraties van ADMA, arginine en SDMA werden gemeten op baseline en tijdens de vierde en 13e cyclus.

Oraal 17β-oestradiol alleen verlaagde ADMA concentraties met 7,7% en de combinatie behandeling verlaagde ADMA concentraties met 7,5% in vergelijking met baseline en placebo na 13 cycli. Beide behandelingen verlaagden ook de arginine concentraties significant in vergelijking met placebo. De SDMA concentraties werden alleen verlaagd door 17β-oestradiol zonder gestodeen. Samenvattend, de behandeling met de combinatie van 17β-oestradiol met gestodeen had geen ander effect op ADMA concentraties dan de behandeling met 17β-oestradiol alleen.

Tot slot wordt in Hoofdstuk 5 het effect van oraal 17β-oestradiol gecombineerd met norethisteron acetaat op ADMA concentraties beschreven. Na 52 weken behandeling met deze combinatie werd een ongeveer even grote daling gezien in ADMA concentraties als werden gevonden met 17β-oestradiol alleen (Hoofdstukken 3 en 4) of met 17β-oestradiol gecombineerd met dydrogesteron (Hoofdstuk 3) of met gestodeen (Hoofdstuk 4). Deze beide progestagenen hadden geen invloed op het effect van 17β-oestradiol op ADMA concentraties. Het is waarschijnlijk dat het toevoegen van norethisteron acetaat aan 17β-oestradiol therapie geen ander effect op ADMA concentraties heeft dan 17β-oestradiol alleen.

ADMA en verschillende toedieningsvormen voor HT

In dezelfde studie die beschreven wordt in Hoofdstuk 4, werd ook het effect van transdermale toediening vergeleken met orale toediening van 17β-oestradiol. Na orale toediening werd een grotere daling in ADMA concentraties gezien dan na transdermale toediening. Oraal 17β-oestradiol verlaagde de SDMA concentraties in vergelijking met placebo, terwijl transdermale 17β-oestradiol toediening geen effect had.
Het verschil in invloed op ADMA concentraties van intranasaal 17β-oestradiol gecombineerd met norethisteron (acetaat) ten opzichte van de orale toedieningsvorm van dezelfde combinatie wordt beschreven in Hoofdstuk 5. Gezonde postmenopauzale vrouwen kregen dagelijks intranasaal of oraal een combinatie van 17β-oestradiol met norethisteron (acetaat) in vergelijkbare doses in een gerandomiseerde, dubbelblinde studie. Nuchtere plasma ADMA, arginine en SDMA concentraties werden gemeten op baseline, week twaalf en week 52.

Na 52 weken waren ADMA concentraties gedaald met 7,4% met orale 17β-oestradiol toediening, terwijl met intranasale 17β-oestradiol toediening geen effect gezien werd (0,8%). Na twaalf weken waren arginine concentraties kortdurend gedaald in beide groepen met ongeveer 6% in vergelijking tot baseline. Oraal 17β-oestradiol met norethisteron acetaat verlaagde ook de SDMA concentraties. De resultaten van deze studies duiden erop dat zowel de transdermale als de intranasale toediening van HT minder effectief zijn in het verlagen van ADMA en SDMA concentraties dan de orale toedieningsvorm.

ADMA en alternatieven van HT

Hoofdstuk 6 gaat over de effecten van een supplement dat zowel soja isoflavonen als Actaea racemosa L. bevat op enkele HVZ risicomarkers in symptomatische vrouwen in de overgang. Vrouwen kregen twaalf weken lang dagelijks placebo of een supplement dat soja isoflavonen en Actaea racemosa L. bevat in een gerandomiseerde, placebo-gecontroleerde, dubbelblinde studie. ADMA, lipiden en CRP concentraties werden gemeten op baseline en in week twaalf in nuchter afgenomen bloedmonsters.

In de groep die het supplement kreeg werd een kleine daling gezien in totaal en LDL-cholesterol na twaalf weken (beide -0,2 mmol/L). Concentraties van ADMA, triglyceriden, lipoproteïne(a) en CRP veranderden niet. Na twaalf weken suppletie werd er geen verschil gevonden tussen de placebo en supplement groepen in effect op de onderzochte risicomarkers.

Twaalf weken suppletie met soja isoflavonen en Actaea racemosa L. had weinig tot geen invloed op de onderzochte HVZ risicomarkers. Dit supplement werkt waarschijnlijk noch beschermend noch ongunstig ten aanzien van het risico van HVZ. Om dit te kunnen bewijzen zijn er grote lange-termijn studies nodig.

In dezelfde studie die beschreven is in Hoofdstuk 6 is gekeken naar het effect van hetzelfde supplement op overgangssymptomen en de resultaten hiervan worden besproken in Hoofdstuk 7. De gemodificeerde Kupperman Index, de Greene Climacteric Scale, een visueel analoge schaal ontworpen om de kwaliteit van leven te meten en het dagelijks aantal en de ernst van de opvliegers werden geëvalueerd tijdens de screening en na zes en twaalf weken.

De scores van al deze symptoomlijsten verbeterden zowel na zes als na twaalf weken in vergelijking met de baseline waarden in beide groepen. Er was echter geen significant verschil in de grootte van verandering in de verschillende scores tussen de placebo en supplement groepen. De combinatie van soja isoflavonen en Actaea racemosa L. gaf niet meer verlichting van overgangsklachten dan placebo bij vrouwen die tenminste vijf opvliegers per dag hebben.
Er is ook gekeken naar de korte termijn effecten van de SERM HMR 3339 op ADMA, arginine en SDMA concentraties in vergelijking met placebo en raloxifene. Dit onderzoek wordt beschreven in Hoofdstuk 8. Postmenopauzale vrouwen kregen twaalf weken lang dagelijks placebo of HMR 3339 2,5 mg of HMR 3339 10 mg of HMR 3339 50 mg of raloxifene 60 mg in een gerandomiseerde, placebo-gecontroleerde, dubbel-blinde, studie. Nuchtere plasma concentraties van ADMA, arginine en SDMA werden gemeten op baseline en na vier en twaalf weken.

Er werd een dosis afhankelijke daling in ADMA en SDMA concentraties gevonden na behandeling met HMR 3339 en de grootste daling werd gezien met 50 mg HMR 3339 in vergelijking met baseline en placebo (7,0% daling in ADMA concentraties en 16,2% daling in SDMA concentraties, beiden na twaalf weken). Twaalf weken behandeling met 60 mg raloxifene verlaagde de SDMA concentraties ten opzichte van placebo maar had geen effect op ADMA concentraties. Arginine concentraties werden door geen van de behandelingen beïnvloed.

Deze resultaten suggereren dat HMR 3339 een potentieel gunstig effect heeft ten aanzien van het risico van HVZ in postmenopauzale vrouwen door het verlagen van de NOS remmer ADMA.

**Conclusie**

Tot slot worden in Hoofdstuk 9 de resultaten van de studies in dit proefschrift besproken en vergeleken met de bestaande literatuur. Verder wordt kort ook de betekenis van deze bevindingen voor de klinische praktijk besproken en er worden aanbevelingen gedaan voor toekomstig onderzoek. Zowel endogene oestrogenen als 17β-oestradiol therapie als de SERM HMR 3339 beïnvloedde ADMA concentraties in vrouwen van middelbare leeftijd. Het toevoegen van het progestageen trimetaston aan 17β-oestradiol therapië verhoogde de daling in ADMA concentraties ten opzichte van 17β-oestradiol alleen. Het toevoegen van hydrogesteron of gestodeen aan 17β-oestradiol therapië had geen invloed op het effect van 17β-oestradiol op ADMA concentraties. Verder leek ook de toevoeging van norethisteron acetaat geen invloed te hebben op het effect van 17β-oestradiol op ADMA concentraties. Zowel de transdermale als intranasale toedieningsvorm van HT was minder effectief in het verlagen van ADMA en SDMA concentraties, dan de orale toedieningsvorm van HT. Een supplement van soja isoflavonen met *Actaea racemosa* L. en een behandeling met de SERM raloxifene hadden geen invloed op ADMA concentraties.

Er bestaat een duidelijk verband tussen zowel endogene oestrogeen concentraties als 17β-oestradiol therapië met ADMA concentraties. Hogere oestrogeen concentraties gaan gepaard met lagere ADMA concentraties in vrouwen. De klinische consequenties van dit verband tussen oestrogenen en ADMA zijn nog niet duidelijk. Ook het mechanisme van de door oestrogenen geïnduceerde ADMA verlaging is nog niet geheel opgehelderd. Onderzoek in de toekomst zou zich met name moeten richten op deze twee aspecten van het verband tussen oestrogenen en ADMA in vrouwen.
List of publications


(5) Marieke O. Verhoeven, Majoie Hemelaar, Tom Teerlink, Peter Kenemans, Marius J. van der Mooren. Effects of intranasal versus oral hormone therapy on asymmetric dimethylarginine in healthy postmenopausal women: a randomized study. Atherosclerosis, in press.

(6) Marieke O. Verhoeven, Tom Teerlink, Peter Kenemans, Sonja D. Zuijdegeest-van Leeuwen, Marius J. van der Mooren. Effects of a supplement containing isoflavones and Actaea racemosa L. on asymmetric dimethylarginine, lipids and C-reactive protein in menopausal women. Fertility and Sterility, in press.

(8) **Marieke O. Verhoeven**, Marius J. van der Mooren, Tom Teerlink, René H.M. Verheijen, Peter G. Scheffer, Peter Kenemans. The influence of physiological and surgical menopause on coronary heart disease risk markers. *Submitted for publication.*
Authors’ affiliations

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- **Sonja D. Zuijdgeest-van Leeuwen**: Numico Research BV, Wageningen, The Netherlands
Curriculum Vitae


Midzomer 2006 getrouwd met Norbert Ligterink.
Als de laatste promovenda van de projectgroep “De Ouder Wordende Vrouw” heb ik de deur van de onderzoekskamers gesloten. Daar is jarenlang hard gewerkt door vele van mijn collega’s. Samen hebben wij lief en leed gedeeld. Het werk had hoogte- en dieptepunten, het was erg prettig om dit te kunnen delen met gelijkgestemde en invloedende collega’s.

Ik wil Marinka Post en Tatjana Vogelvang bedanken voor het warme welkom in de groep en het vele lezen en corrigeren van mijn schrijfsels. Verder wil ik ze graag bedanken voor het rotvaste geloof in mij dat mijn boekje er ook wel zou komen. Tatjana wil ik speciaal bedanken voor het inwerken en het aandragen van oplossingen. Marinka wil ik speciaal bedanken dat ze altijd voor mij klaar stond, zelfs tijdens haar opleiding heeft zij belangeloos samen met mij in de vriezerruimte in de kelder bloedmonster staan uitzoeken.

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Professor Peter Kenemans was de drijfveer achter de groep en het onderzoek. Hij genereerde de gunstige randvoorwaarden en gaf de impetus aan het onderzoek rond menopauze en hart- en vaatziekten. Zonder hem zou het fundament hebben ontbroken, en dit proefschrift nooit geschreven zijn. Op een persoonlijk vlak heb ik

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Tot slot wil ik Norbert Ligterink bedanken voor zijn geduld, hulp en liefde in het algemeen maar zeker ook tijdens het laatste stuk van het productieproces van dit boekje.
### List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. racemosa L.</td>
<td>Actaea racemosa linnaeus</td>
</tr>
<tr>
<td>ACE-inhibitors</td>
<td>angiotensin converting enzyme inhibitor</td>
</tr>
<tr>
<td>Actaea racemosa L.</td>
<td>Actaea racemosa linnaeus</td>
</tr>
<tr>
<td>ADMA</td>
<td>asymmetric dimethylarginine</td>
</tr>
<tr>
<td>AN(C)OVA</td>
<td>analysis of (co)variance</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>CEE</td>
<td>conjugated equine (o)estrogens</td>
</tr>
<tr>
<td>CHD</td>
<td>coronary heart disease</td>
</tr>
<tr>
<td>CI</td>
<td>confidence interval</td>
</tr>
<tr>
<td>CV</td>
<td>coefficient of variation</td>
</tr>
<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>DDAH</td>
<td>dimethylarginine dimethylaminohydrolase</td>
</tr>
<tr>
<td>$E_2$</td>
<td>(o)estradiol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FMD</td>
<td>flow mediated dilatation</td>
</tr>
<tr>
<td>FSH</td>
<td>follicle-stimulating hormone</td>
</tr>
<tr>
<td>HDL</td>
<td>high-density lipoprotein</td>
</tr>
<tr>
<td>HELLP syndrome</td>
<td>haemolysis, elevated liver enzymes and low platelets’ syndrome</td>
</tr>
<tr>
<td>HMR 3339</td>
<td>4-chloro-11β-(4-(2-(diethylamino)ethoxy)phenyl)-estra-1,3,5(10)-triene-3, 17β-diol</td>
</tr>
<tr>
<td>HMR 2.5</td>
<td>HMR 3339 2.5 mg per day group</td>
</tr>
<tr>
<td>HMR 10</td>
<td>HMR 3339 10 mg per day group</td>
</tr>
<tr>
<td>HMR 50</td>
<td>HMR 3339 50 mg per day group</td>
</tr>
<tr>
<td>HUS</td>
<td>haemolytic uraemic syndrome</td>
</tr>
<tr>
<td>HT</td>
<td>hormone therapy</td>
</tr>
<tr>
<td>IMT</td>
<td>intima-media thickness</td>
</tr>
<tr>
<td>LDL</td>
<td>low-density lipoprotein</td>
</tr>
<tr>
<td>M</td>
<td>menopause</td>
</tr>
<tr>
<td>M - 2</td>
<td>2 years before menopause</td>
</tr>
<tr>
<td>M - 1</td>
<td>1 year before menopause</td>
</tr>
<tr>
<td>M + 1</td>
<td>1 year after menopause</td>
</tr>
<tr>
<td>M + 2</td>
<td>2 years after menopause</td>
</tr>
<tr>
<td>NET(A)</td>
<td>norethisterone (acetate)</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>oE₂</td>
<td>oral micronised 17β-(o)estradiol</td>
</tr>
<tr>
<td>oE₂ + D</td>
<td>oE₂ continuously combined with dydrogesterone</td>
</tr>
<tr>
<td>oE₂ + G</td>
<td>oE₂ continuously combined with gestodene</td>
</tr>
<tr>
<td>oE₂ + T</td>
<td>oE₂ continuously combined with trimegestone</td>
</tr>
<tr>
<td>pBSO</td>
<td>prophylactic bilateral salpingo-oophorectomy</td>
</tr>
<tr>
<td>PRMTs</td>
<td>protein arginine methyltransferases</td>
</tr>
<tr>
<td>raloxifene 60</td>
<td>raloxifene 60 mg per day group</td>
</tr>
<tr>
<td>SDMA</td>
<td>symmetric dimethylarginine</td>
</tr>
<tr>
<td>SERM</td>
<td>selective estrogen receptor modulator</td>
</tr>
<tr>
<td>sE-selectin</td>
<td>soluble E-selectin</td>
</tr>
<tr>
<td>sICAM-1</td>
<td>soluble intercellular adhesion molecule-1</td>
</tr>
<tr>
<td>SPSS</td>
<td>Statistical Package for the Social Sciences</td>
</tr>
<tr>
<td>SUPPL</td>
<td>supplement containing soy isoflavones extract and Actaea racemosa L.</td>
</tr>
<tr>
<td>Supplement</td>
<td>supplement containing soy isoflavones extract and Actaea racemosa L.</td>
</tr>
<tr>
<td>sVCAM-1</td>
<td>soluble vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>tE₂</td>
<td>transdermal 17β-oestradiol</td>
</tr>
<tr>
<td>TTP</td>
<td>thrombotic thrombocytopenic purpura</td>
</tr>
<tr>
<td>VAS</td>
<td>visual analogue scale</td>
</tr>
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