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The effect of sequential three-monthly hormone replacement therapy on several cardiovascular risk estimators in postmenopausal women*

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Objective: To investigate the changes in plasma lipids and lipoproteins, low-density lipoprotein (LDL) oxidizability, and plasma homocysteine during postmenopausal sequential 3-monthly hormone replacement therapy.

Design: Open longitudinal prospective study.

Setting: Gynecological outpatient department of a university hospital.

Patient(s): Thirty-nine healthy nonhysterectomized postmenopausal women.

Intervention(s): Oral conjugated estrogen, 0.625 mg/d, combined with oral medrogestone 10 mg/d during the last 14 days of each 84-day treatment cycle. The treatment was given for four treatment cycles of 84 days (1 year).

Main Outcome Measure(s): Plasma lipids and lipoproteins, LDL oxidizability, and plasma homocysteine.

Result(s): After 1 year of treatment plasma concentrations of total cholesterol and LDL cholesterol were 3.5% and 8.7% lower, respectively. High density lipoprotein cholesterol, apolipoprotein A-I, and triglycerides were 6.5%, 9.0% and 16% higher, respectively. Apolipoprotein B concentration remained unchanged. The results on LDL oxidizability were inconsistent. Plasma homocysteine decreased with 12.3% during the first 6 months of treatment in women with higher homocysteine concentrations at baseline. These values returned to baseline levels during the second half year of treatment.

Conclusion(s): This sequential hormone regimen induced beneficial changes in the conventional lipid and lipoprotein risk estimators, whereas the observed changes in the other markers remained inconclusive and/or of minor importance. *Fertil Steril*® 1997;67:67-73

Key Words: Postmenopause, estrogen, progestogen, lipids, lipoproteins, LDL oxidizability, homocysteine

Hormone replacement therapy (HRT) is known to reduce vasomotor symptoms, genital atrophy, and several atypical complaints (e.g., sleeplessness, tiredness, painful joints, and depressive mood changes)

in postmenopausal women (1, 2). Furthermore, it has been reported to reduce the incidence of osteoporotic fractures (2) and of cardiovascular disease (2, 3). To accomplish the latter two preventive effects of HRT, long-term supplementation is recommended in selected groups of subjects at increased risk (2).

To prevent the development of endometrial hyperplasia and carcinoma in nonhysterectomized women the addition of progestogen to the estrogen administration is recommended (4). A disadvantage of sequential estrogen-progestogen therapy, however, is that it restores vaginal bleeding, which is an important reason for poor compliance in many women (5). Therefore, alternative treatment regimens, such as continuous combined estrogen/progestogen regimen and 3-monthly progestogen administration during estrogen supplementation (6, 7), currently are

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being investigated to reduce the frequency and severity of the bleeding episodes, thereby improving patient compliance.

Until now, cardiovascular consequences of such alternative hormone therapies have not been published. Reducing the frequency of progestogen administration may reduce the reported adverse effect of progestogens on the lipid profile (8) and therefore be beneficial with respect to the cardiovascular risk. However, it may also increase the risk of developing endometrial hyperplasia (6, 7). Therefore, this alternative HRT regimen needs more research before it can be considered for general use.

To approximate the influence of HRT regimen with respect to the risk of developing cardiovascular disease (CVD) it is common practice to study the changes in plasma lipids and lipoproteins. However, these changes only partly contribute to the preventive effects of HRT on CVD risk in postmenopausal women (9).

New risk estimators of great interest are the size of low-density lipoprotein (LDL) particles and their susceptibility to oxidation *in vitro*. It has been reported that small LDL particles (10) as well as oxidized LDL particles (11) have greater atherogenic properties than native LDL. Furthermore, small LDL particles are more susceptible to oxidation (12). For LDL oxidation three parameters are relevant (13): [1] the lag time, [2] the oxidation rate, and [3] the maximal amount of dienes produced at the end of the final oxidation. The lag time is the time needed to obtain a significant increase in lipid peroxidation products (dienes) and is considered a function of the amount of antioxidants present in the LDL. The rate at which lipid peroxidation occurs is another relevant parameter of LDL oxidizability. Finally, lipid peroxidation stops because of the lack of substrate: the total amount of dienes produced is the third relevant parameter of LDL oxidizability. Exogenous hormones may influence LDL oxidizability (14).

Homocysteine has been found to be another important risk factor for CVD independent of plasma lipid profiles (15). Its reported increase after menopause has been suggested to explain part of the increased risk of developing CVD in postmenopausal women (16). The recently published data on decreased homocysteine levels during HRT (17) may indeed partially account for the decreased risk in CVD during postmenopausal HRT.

This study was designed to investigate the effects of 3-monthly progestogen administration during estrogen supplementation as an alternative for the usually practiced monthly administration. This article reports the effects of this regimen on plasma lipids and lipoproteins, parameters of LDL oxidizability, and plasma homocysteine.

MATERIALS AND METHODS

Patients

This study was approved by the Institutional Review Board of the University Hospital Nijmegen. The participating women were recruited by advertisement in a local newspaper. Selected subjects were healthy postmenopausal nonhysterectomized women aged 45 to 60 years who gave their written informed consent on beforehand. They were amenorrhoic for at least 6 months and their serum FSH concentrations were higher than 36 mIU/mL (conversion factor to SI unit, 1.00). No estrogen or progestogen therapy was used within 90 days before screening. Excluded were women with a history or active presence of any contraindication for HRT. In addition, also excluded were women with a systolic blood pressure higher than 160 mm Hg and diastolic blood pressure higher than 100 mm Hg, an endocrine disease except for controlled thyroid disease, and total cholesterol concentrations higher than 7.5 mmol/L. Women with any malignancy except for successfully resected basal cell skin cancer, malabsorption disorders, obesity (exceeding 20% of ideal range for weight and height), a smoking habit, alcohol or drug abuse, or malignant changes on the prestudy mammogram were not included. Furthermore, use of lipid-lowering agents, dopaminergic or antidopaminergic drugs, niacin, clonidine, and tibolone within 30 days of the screening was not permitted. None of the women reported supplementation of vitamins related to homocysteine metabolism, such as vitamin B₆, B₁₂ and folate supplements.

Beside medical history, the screening consisted of physical and gynecologic examination, including Papanicolaou smear and endometrial biopsy by microcurette (Endocurette; Farina Lanfranco, Venezia, Italy), mammography (unless performed within 6 months before screening), routine laboratory hematology, blood chemistry, and urinalysis determinations. Furthermore, FSH and lipid and lipoproteins were assayed. At baseline, *i.e.*, between 7 and 21 days after screening, the lipid and lipoproteins were quantified again to assess possible fluctuations in time. Also, plasma samples were taken for homocysteine assays and then the study treatment was started. Baseline values were used in calculations and shown in Tables 1 to 4. On cycle days 82 through 84 of the 84-day cycles two and four, the endometrial biopsy was repeated and blood samples were drawn for determination of lipids, lipoproteins, and homocysteine. At cycle four the Papanicolaou smear, the mammography, routine laboratory hematology, blood chemistry, and urinalysis were repeated.

After screening, 41 women were included. One woman was considered a screening failure because

a mammographic lesion suspect for carcinoma was detected before study entry. There were three drop-outs. One woman was excluded in cycle one from further study because of bad compliance. Furthermore, two women were excluded in cycle three, one subject because of unspecific fatigue and tender breasts, and the other because of progressive headaches.

All women were treated daily with conjugated estrogen (0.625 mg/d, Premarin®; Wyeth Laboratories, Hoofddorp, The Netherlands), for four treatment cycles of 84 days and with medrogestone (10 mg/d, Colpro®; Wyeth Laboratories) during the last 14 days of each 84-day treatment cycle.

Blood Sampling

Venous blood was obtained in evacuated collection tubes for FSH and blood chemistry (Corvac®, Sherwood Medical, St. Louis, MO) after a 12-hour fast. A 15% solution of 15.0 mg K₃-EDTA (Sherwood Medical, Ballymoney, Northern Ireland) was added to blood drawn for analysis of hematologic parameters, lipids, lipoproteins, and homocysteine. Plasma was isolated or separated within 1 to 2 hours after blood sampling. Serum concentrations of FSH were measured with a specific immunoradiometric assay (Medgenix, Fleurus, Belgium).

Lipids and Lipoproteins

Very low-density lipoproteins were isolated from plasma within 5 days after blood sampling by ultracentrifugation at $d = 1.006$ g/mL using a Kontron TFT 45.6 rotor for 16 hours at $168,000 \times g$ at 14°C in a Beckman L7-55 ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA). High-density lipoprotein cholesterol (HDL-C) in stored plasma samples was determined weekly by the polyethylene glycol 6000 method. For HDL-C the interassay coefficient of variation (CV) amounted to 2.3% ($n = 20$). Plasma total cholesterol and triglycerides (TG) were measured by enzymatic methods using commercially available reagents (CHOD-PAP cholesterol reagent, cat. no. 237574; Boehringer Mannheim, Mannheim, Germany, and SERA-PAK TG cat. no. 6684; Miles, Milan, Italy). Both measurements were performed with a centrifugal analyzer (Multistat III; Instrumentation Laboratory, Lexington, MA). Interassay CVs were 1.7% and 1.5%, respectively ($n = 20$). The accuracy check for cholesterol against an Abell-Kendall method approved by the Centers for Disease Control (CDC; Atlanta, GA) was within 3% of target values. Low-density lipoprotein cholesterol (LDL-C) was calculated by subtracting the cholesterol content in the $d < 1.006$ g/mL fraction and in the HDL fraction from total plasma cholesterol. Plasma sam-

ples used for apolipoproteins and total homocysteine determinations were stored at -80°C until the end of the study. To minimize the imprecision all samples from the same subject were analyzed in duplicate in the same run. Apolipoprotein (Apo) A-I and B were measured by immunonephelometry; accuracy was adjusted to CDC criteria on the base of an exchange of sera.

Low-Density Lipoprotein Isolation and In Vitro Oxidation

Plasma was separated from the blood within 1 to 2 hours and aliquots were stored at -80°C in the presence of 6 g saccharose/L. To minimize analytical variation, all samples of the same subject were analyzed in the same run. Low-density lipoproteins were isolated by a short-run ultracentrifugation method and were thereafter tested for their susceptibility to copper-induced LDL oxidation in vitro (18). Briefly, after isolation and washing by subsequent ultracentrifugation for 16 hours at $168,000 \times g$, the LDL was dialyzed for 24 hours in the dark at 4°C against phosphate-buffered saline (pH 7.4), containing 10 $\mu\text{mol/L}$ EDTA. The buffer was made oxygen-free by vacuum degassing, followed by purging with nitrogen. The LDL-containing sample was filtered through a 0.45- μm filter and diluted with dialysis buffer to a final concentration of 0.05 mg LDL-protein/mL and 10 μmol EDTA/L. The oxidation was initiated by the addition of a freshly prepared solution (15 $\mu\text{mol/L}$) of copper chloride (CuCl_2). The kinetics of LDL oxidation were determined by monitoring the change in the 234-nm diene absorption on an UV-spectrophotometer (Lambda 5; Perkin-Elmer, Norwalk, CT), equipped with a six-position automatic sample changer, at 30°C. The change of absorbance at 234-nm versus time was divided into three consecutive phases, i.e., a lag phase, a propagation phase, and a decomposition phase. Oxidation parameters (lag time, maximal rate of oxidation, and total amount of conjugated dienes formed during oxidation) were calculated as described previously (18).

Fatty Acids in LDL

The LDL samples (0.4 mL) were saponified by incubation with 1.6 mL of 0.3 mol NaOH/L in 90% ethanol (vol/vol) at 37°C for 1 hour. To 0.4 mL of the mixture, 0.4 mL of water and 50 μL of 12 mol HCl/L was added. The fatty acids were extracted twice with 2 mL n-hexane. The pooled organic phase was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 100 μL of ethanol containing 400 $\mu\text{mol/L}$ of heptadecanoic acid as an internal standard and derivatized to 4-nitrophenylhydrazides as described elsewhere (19). To the hy-

drazides mixture 0.2 mL of 0.5 mol/L Tris (pH 10.0) and 2 mL of n-hexane was added. After vortexing for 30 seconds and centrifugation ($1,500 \times g$ for 5 minutes) the top n-hexane layer was evaporated under a stream of nitrogen at room temperature. This procedure was repeated twice. The residue was dissolved in 800 μ L of absolute methanol, and a 2- μ L aliquot was analyzed for fatty acids by high-performance liquid chromatography (HPLC; Spectra Physics, Eindhoven, The Netherlands). A ChromSep column packed with Chromspher C8 (20×100 mm; Chrompack, Middelburg, the Netherlands) was used to elute the fatty acids with 80% (vol/vol) acetonitrile (pH adjusted to 4.5 with sulfuric acid) at a flow rate of 0.25 mL/min. The absorbance at 390 nm was measured with a Spectra Physics (SP 8450) spectrophotometer (Spectra Physics, Eindhoven, the Netherlands). For technical reasons the fatty acid measurement in LDL both at baseline and cycle two was performed in only 27 women.

Vitamin E

The vitamin E concentrations in LDL were determined by HPLC as described previously (12). For technical reasons the vitamin E measurement both at baseline and cycle two was performed in only 19 women.

Homocysteine

Total homocysteine was measured in plasma, stored at -80°C until the end of the study, by HPLC followed by fluorimetric detection according to Fiskerstrand et al. with some modifications (20). The detection limit was 0.5 μ mol/L and the intra- and interassay coefficient of variation amounted both to $< 5\%$.

Statistics

Unless indicated statistical "endpoint analyses" were performed on data of 39 women, of whom at least data at cycle two were available, using the "last visit carried forward" procedure. All statistical comparisons were performed with the Friedman two-way analysis of variance, and the Wilcoxon's signed-rank test. A P value < 0.05 was considered to be statistically significant. Statistical analyses were performed with the Dyna-stat computer program (Dynamic Microsystems, Inc., Washington, D.C.).

RESULTS

Study Population

Characteristics of the study population are given in Table 1. During the treatment period no clinically

Table 1 Characteristics of the Study Population*

	Baseline	Cycle 4
Age (y)	52.6 \pm 3.5	
Amenorrhea (mo)	41.7 \pm 32.1	
Body mass index (kg/m ²)	24.0 \pm 2.6	23.7 \pm 2.5†
Blood pressure (mm Hg)		
Systolic	130 \pm 17	130 \pm 15
Diastolic	83 \pm 9	80 \pm 8†

* Values are means \pm SD ($n = 39$; except for cycle 4; $n = 38$).

† $P < 0.05$ (Wilcoxon's signed rank test; comparing cycle 4 with baseline).

relevant changes were found in body mass index and blood pressure.

Lipids and (Apo)lipoproteins

Fasting plasma concentrations of lipids and lipoproteins at baseline and on cycle days 82 through 84 of cycles two and four are given in Table 2. Between screening and baseline no significant differences were detected in the plasma lipids and lipoproteins, except for a 10.1% difference ($P = 0.04$) in the triglycerides (screening: 1.19 ± 0.56 mmol/L; baseline: 1.07 ± 0.37 mmol/L). Compared with baseline values, total cholesterol and LDL-C were 3.5% ($P < 0.05$) and 8.7% ($P < 0.01$) lower, respectively, whereas HDL-C and TG were 6.5% ($P < 0.05$) and 16% ($P < 0.01$) higher, respectively. Parallel to HDL-C, Apo A-I was 9.0% ($P < 0.0001$) higher, whereas Apo B remained unchanged. Furthermore, the atherogenic indices LDL-C/HDL-C and Apo B/Apo A-I were 13% ($P < 0.001$) and 11% ($P < 0.0001$) lower, respectively.

LDL Oxidation

The LDL oxidation characteristics showed minor changes after two cycles of treatment (6 months): lag time decreased from 109 ± 24 to 105 ± 22 minutes (mean \pm SD) ($P < 0.05$), oxidation rate decreased from 9.6 ± 1.6 to 9.3 ± 1.7 nmol dienes/min per milligram LDL protein (mean \pm SD), and the maximum amount of produced dienes decreased from 552 ± 65 to 530 ± 59 nmol/mg protein ($P < 0.01$). Table 3 shows data of fatty acid composition and vitamin E contents in LDL. The percentages of palmitic acid, stearic acid, oleic acid, and linoleic acid, and the vitamin E concentration changed significantly.

Homocysteine

Fasting plasma homocysteine was 6.7% lower ($P < 0.05$) in cycle two, but returned to baseline level in cycle four (Table 4). When dividing the study population into women with lower plasma homocysteine

Table 2 Plasma Concentrations of Lipids, (Apo)lipoproteins and Ratios*

	Baseline	Cycle 2	Cycle 4	P†
Total cholesterol	5.69 ± 0.76	5.38 ± 0.77§	5.49 ± 0.80‡	<0.01
HDL cholesterol	1.53 ± 0.30	1.57 ± 0.30	1.63 ± 0.30‡	<0.05
LDL cholesterol	3.80 ± 0.75	3.40 ± 0.78	3.47 ± 0.85§	<0.01
VLDL cholesterol	0.36 ± 0.20	0.41 ± 0.22	0.39 ± 0.20	<0.05
Triglycerides†	1.07 ± 0.37	1.30 ± 0.54	1.24 ± 0.45§	<0.001
VLDL triglycerides	0.60 ± 0.35	0.64 ± 0.39	0.58 ± 0.31	NS
Apolipoproteine A-I	1511 ± 173	1611 ± 203	1647 ± 187	<0.001
Apolipoproteine B	1130 ± 195	1104 ± 216	1106 ± 206	NS
Ratios				
LDL-cho/HDL-cho	2.61 ± 0.74	2.27 ± 0.80	2.25 ± 0.81	<0.001
Apo B/Apo A-I	0.76 ± 0.17	0.70 ± 0.16	0.68 ± 0.17	<0.001

* Values are means ± SD (n = 39; except for cycle 4; n = 38). Concentrations are expressed in mmol/L (except apolipoproteins in mg/L).

† Friedman's two-way analysis of variance (testing for differences between baseline, cycle 2 and cycle 4), followed by Wilcoxon's signed rank test (testing for differences versus baseline). NS, not significant.

‡ P < 0.05.

§ P < 0.01.

|| P < 0.001.

concentration (Lower group: baseline homocysteine < 13.3 μmol/L) and women with higher plasma homocysteine concentration (Higher group: baseline homocysteine > 13.3 μmol/L), only the Higher group showed significantly lower concentrations in cycle two (-12.3 %, P < 0.01), but no significant difference was detected between cycle four and baseline. No significant differences were found in the Lower group.

DISCUSSION

In nonhysterectomized women the sequential addition of a progestogen to the estrogen replacement therapy induces vaginal bleeding, which impairs the

Table 3 Fatty Acid Composition and Vitamin E Content of LDL in Plasma*

	Baseline	Cycle 2
Palmitic acid (%)	21.5 ± 1.7	23.9 ± 2.5§
Stearic acid (%)	6.3 ± 0.4	5.9 ± 0.4§
Oleic acid (%)	18.2 ± 2.2	19.2 ± 2.5‡
Linoleic acid (%)	46.6 ± 3.4	43.3 ± 4.6§
Arachidonic acid (%)	7.4 ± 1.5	7.7 ± 1.6
Vitamin E (μmol/L)	11.8 ± 4.4	10.0 ± 2.8†
Vitamin E/linoleic acid (nmol/μmol)	5.6 ± 1.1	5.5 ± 0.7
Vitamin E/PUFA (nmol/μmol)	4.9 ± 1.0	4.7 ± 0.5
Vitamin E/apo B (nmol/mg)	11.6 ± 2.7	10.6 ± 1.4
PUFA/total fatty acids (%)	55.6 ± 2.6	54.9 ± 3.9

* Values are mean ± SD (n = 27; except vitamin E and ratios; n = 19). Statistics were performed with Wilcoxon's signed rank test (comparing cycle 2 with baseline).

† P < 0.05.

‡ P < 0.01.

§ P < 0.001.

|| PUFA, poly unsaturated fatty acids.

Table 4 Plasma Concentrations of Total Homocysteine*

	Baseline	Cycle 2	Cycle 4	P†
Total group‡	13.3 ± 2.5	12.4 ± 2.5	13.3 ± 3.1	NS
Higher-group§	15.4 ± 2.0	13.5 ± 2.6**	14.8 ± 2.3	NS
Lower-group	11.4 ± 1.0	11.4 ± 2.2	12.0 ± 3.2	NS

* Values are means ± SD; Concentrations in μmol/L.

† Friedman's two-way analysis of variance (testing for differences between baseline, cycle 2 and cycle 4), followed by Wilcoxon's signed rank test (testing for differences versus baseline). NS, not significant.

‡ Total group: n = 23.

§ Higher group: total homocysteine > 13.3 μmol/L (n = 11).

|| Lower group: total homocysteine < 13.3 μmol/L (n = 12).

¶ P < 0.05.

** P < 0.01.

acceptance of, and compliance with, combined HRT (5). Alternative treatment regimen, as continuous combined estrogen-progestogen regimen and 3-monthly progestogen administration during estrogen supplementation may improve patient compliance by reducing the frequency and severity of bleeding. Reduced frequency of progestogen administration has been reported to offer not only an acceptable cycle control (6), but also an increased tendency to develop endometrial hyperplasia (6, 7). Long-term safety studies with sequential 3-monthly estrogen-progestogen combinations are needed to investigate the relevancy of such treatment for routine use.

Until now, cardiovascular consequences of sequential 3-monthly HRT have not been published. It was hypothesized that reduced frequency of progestogen administration may reduce the reported adverse effect of progestogens on the lipid profile (8) and therefore be beneficial with respect to cardiovascular risk. This 3-monthly HRT regimen was evaluated concerning the changes in several important cardiovascular risk factors. Special interest was given to new cardiovascular risk estimators, because there is no doubt that lipids and lipoproteins explain only part (9) of the cardioprotective influence of HRT (2, 3).

It was shown that most plasma lipids and lipoproteins, except for TG, underwent beneficial changes with respect to the risk of developing CVD. This finding is in concordance with many studies that investigated the effects of HRT on plasma lipids and lipoproteins. When compared with sequential regimen of conjugated estrogen and monthly medrogestone (5 mg/d) (21), the 3-monthly administration of medrogestone (10 mg/d) showed more estrogenic effects, as is apparent from the increase in HDL-C and apo A-I (and TG) and the decrease in total cholesterol and LDL-C. It appears therefore that reducing the frequency of progestogen administration may benefit the lipid profile and so reduce cardiovas-

cular risk. This could be of great importance considering the fact that this alternative HRT regimen was designed to improve compliance, especially for those women in need of long-term treatment to prevent CVD. However, this hypothesis needs further research by prospective randomized studies, because minor variations in different studies over time in different women do not allow this conclusion to be drawn.

Low-density lipoprotein oxidation has been suggested to be an additional important risk factor for atherogenesis. Once LDL is infiltrated in the vascular bed, a supply of antioxidants prevents lipid peroxidation of the polyunsaturated fatty acids within the LDL. Indeed, 17β -E₂ administered by intra-arterial infusion, patch application, or by implantation reduced the lag time of the LDL oxidizability curve (14, 22). In our study we observed a decrease in the lag time of in vitro oxidation with 3.7%, a significant, but probably not a physiological difference because the ratio of vitamin E per milligram Apo B did not change. Furthermore, oxidation rates remained similar, despite a slight but statistically significant decrease in the relative content of linoleic acid, which is sensitive to oxidation in contrast to palmitic acid or oleic acid.

Inherent to the choice of hormone regimen, changes in plasma lipoproteins as well as in LDL oxidizability are small. Previously, we showed that LDL consists of three subfractions: LDL₃ with the highest density was most rapidly oxidized with Cu²⁺ in vitro (12). On the other hand, the LDL subfraction pattern is a function of plasma TG concentration. So, if a HRT regimen is designed with small changes in plasma lipids and lipoproteins, such as the present one, then changes in LDL oxidizability will be minimal. This indeed could be established.

Possible explanations for the difference with our own findings are the different kinds of estrogens used (17β -E₂ versus conjugated estrogens in our study), the route of administration (intra-arterial, transdermal, implantation versus oral administration in our study), and the duration of administration (direct, 3 and 16 weeks versus 6 months in our study), and possibly most important the administration of a progestogen. Progestogens reportedly counteract the beneficial effects of estrogen administration on lipid profiles (8).

Homocysteine is an independent risk factor for CVD (15). It was demonstrated that plasma homocysteine concentrations in postmenopausal women are higher than in premenopausal women and therefore may contribute to the increased cardiovascular risk in postmenopausal women (16). Furthermore, postmenopausal E₂/dydrogesterone administration for 2 years was associated with a decrease in serum

homocysteine, which may contribute to the cardioprotective effect of HRT (17). The recently reported observation by Anker et al. (23) adds to this conclusion. They found a reduction in plasma homocysteine of 29.8% after 9 to 12 months and of 24.5% after 13 to 18 months of treatment with the anti-estrogen tamoxifen, a adjuvant drug that has been associated with reduced cardiovascular mortality, which is given to women with breast cancer. Our present observation during 12 months of conjugated estrogen-medrogestone administration, however, indicates that the reduction in homocysteine may only be temporary. In relation to previous studies we used a relative high dosage of medrogestone, possibly exerting an unfavorable effect on homocysteine metabolism. In agreement with previous observations (17), the decreases in homocysteine, if any, were especially observed in those women having higher homocysteine concentrations, indicating that they may benefit the most of the homocysteine-lowering influence of HRT.

Arteriosclerotic changes related to elevated homocysteine concentrations have been suggested to be induced by increased lipid peroxidation due to reactive oxygen generation by homocysteine (24). Blom et al. (25) failed to confirm this hypothesis in in vitro oxidation studies in hyperhomocysteinemic subjects. In the present study LDL oxidation showed a small but significant increase, not in line with the temporarily decreased homocysteine concentrations.

From the data obtained in this study, it can be concluded that this sequential HRT regimen induced several favorable changes in lipid and lipoprotein concentrations, as well as in homocysteine concentrations, which may counteract the minor undesirable changes in LDL susceptibility to oxidation. Therefore, our data explain in part the reduced risk of developing CVD in postmenopausal women on HRT.

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REFERENCES

1. Notelovitz M. Estrogen replacement therapy: indications, contraindications, and agent selection. *Am J Obstet Gynecol* 1989;161:1832-41.
2. Grady D, Rubin SM, Petitti DB, Fox CS, Black D, Ettinger B, et al. Hormone therapy to prevent disease and prolong life

- in postmenopausal women. *Ann Intern Med* 1992;117:1016-37.
3. Stampfer MJ, Colditz GA. Estrogen replacement therapy and coronary heart disease: a quantitative assessment of the epidemiologic evidence. *Prev Med* 1991;20:47-63.
 4. Creasy GW, Kafrisen ME, Upmalis D. Review of the endometrial effects of estrogens and progestins. *Obstet Gynecol Surv* 1992;47:654-78.
 5. Wren BG, Brown L. Compliance with hormonal replacement therapy. *Maturitas* 1991;13:17-21.
 6. Ettinger B, Selby J, Citron JT, Vangessel A, Ettinger VM, Hendrickson MR. Cyclic hormone replacement therapy using quarterly progestin. *Obstet Gynecol* 1994;83:693-700.
 7. Cerin Å, Heldaas K, Moeller B. Adverse endometrial effects of longcycle estrogen and progestogen replacement therapy. *N Engl J Med* 1996;334:668.
 8. Rijpkema AHM, Van der Sanden AA, Ruijs AHC. Effects of post-menopausal oestrogen-progestogen replacement therapy on serum lipids and lipoproteins: a review. *Maturitas* 1990;12:259-85.
 9. Bush TL, Barrett-Connor E, Cowan LD, Criqui MH, Wallace RB, Suchindran CM, et al. Cardiovascular mortality and non-contraceptive use of estrogen in women: results from the Lipid Research Clinics Program Follow-up Study. *Circulation* 1987;75:1102-9.
 10. Campos H, Genest JJ Jr, Blijlevens E, McNamara JR, Jenner JL, Ordovas JM, et al. Low density lipoprotein particle size and coronary artery disease. *Arterioscler Thromb* 1992;12:187-95.
 11. Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL. Beyond cholesterol: modifications of low density lipoprotein that increase its atherogenicity. *N Engl J Med* 1989;320:915-24.
 12. De Graaf J, Hak-Lemmers HLM, Hectors MPC, Demacker PNM, Hendriks JCM, Stalenhoef AFH. Enhanced susceptibility to in vitro oxidation of the dense low density lipoprotein subfraction in healthy subjects. *Arteriosclerosis* 1991;11:298-306.
 13. Jialal I, Devaraj S. Low-density lipoprotein oxidation, antioxidants, and atherosclerosis: a clinical biochemistry perspective. *Clin Chem* 1996;42:498-506.
 14. Sack MN, Rader DJ, Cannon RO III. Oestrogen and inhibition of oxidation of low-density lipoproteins in postmenopausal women. *Lancet* 1994;343:269-70.
 15. Ueland PM, Refsum H, Brattström L. Plasma homocysteine and cardiovascular disease. In: Francis RB, editor. *Atherosclerotic cardiovascular disease, hemostasis and endothelial function*. New York: Marcel Dekker Inc., 1992:183-236.
 16. Wouters MGAJ, Moorrees MThEC, Van der Mooren MJ, Blom HJ, Boers GHJ, Schellekens LA, et al. Plasma homocysteine and menopausal status. *Eur J Clin Invest* 1995;25:801-5.
 17. Van der Mooren MJ, Wouters MGAJ, Blom HJ, Schellekens LA, Eskes TKAB, Rolland R. Hormone replacement therapy may reduce high serum homocysteine in postmenopausal women. *Eur J Clin Invest* 1994;24:733-6.
 18. Kleinveld HA, Hak-Lemmers HLM, Stalenhoef AFH, Demacker PNM. Improved measurement of low-density lipoprotein susceptibility to copper-induced oxidation; application of a short low-density lipoprotein isolation procedure. *Clin Chem* 1992;38:2066-72.
 19. Miwa H, Yamamoto M, Nishida T, Nunoi K, Kikuchi M. High-performance liquid chromatographic analysis of serum long-chain fatty acids by direct derivatization method. *J Chromatogr* 1987;416:237-45.
 20. Te Poele-Pothoff MTWB, Van den Berg M, Franken DG, Boers GHJ, Jakobs C, De Kroon IFI, et al. Three different methods for the determination of total homocysteine in plasma. *Ann Clin Biochem* 1995;32:218-20.
 21. Van der Mooren MJ, Gevers Leuven JA, Rolland R. Effect of conjugated estrogen with and without medrogestone*: a prospective study. *Maturitas* 1994;19:33-42.
 22. Keaney JF Jr, Shwaery GT, Xu A, Nicolosi RJ, Loscalzo J, Foxall TL, et al. 17 β -Estradiol preserves endothelial vasodilator function and limits low-density lipoprotein oxidation in hypercholesterolemic swine. *Circulation* 1994;89:2251-9.
 23. Anker G, Lønning PE, Ueland PM, Refsum H, Lien EA. Plasma levels of the atherogenic amino acid homocysteine in postmenopausal women with breast cancer treated with tamoxifen. *Int J Cancer* 1995;60:365-8.
 24. Stamler JS, Osborne JA, Jaraki O, Rabbani LE, Mullins M, Singel D, et al. Adverse vascular effects of homocysteine are modulated by endothelium-derived relaxing factor and related oxides of nitrogen. *J Clin Invest* 1993;91:308-18.
 25. Blom HJ, Kleinveld HA, Boers GHJ, Demacker PNM, Hak-Lemmers HLM, Te Poele-Pothoff MTWB, et al. Lipid peroxidation and susceptibility of low-density lipoprotein to in-vitro oxidation in hyperhomocysteinaemia. *Eur J Clin Invest* 1995;25:149-54.
- Note.* Additional references are available from the authors upon request.