*Free Radical Research*, Volume 38 Number 2 (February 2004), pp. 129–137

Taylor & Francis
healthsciences

# Impact of Long-term Hormone Replacement Therapy on *In vivo* and *In vitro* Markers of Lipid Oxidation

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Accepted by Professor J. Vina

*(Received 23 June 2003; In revised form 22 October 2003)*

Postmenopausal hormone replacement therapy (HRT) with estrogen has been suggested to inhibit oxidation of low-density lipoprotein (LDL) *in vitro*, but progestins may oppose this effect. We studied whether estrogen HRT and combined HRT with estrogen and progestin differ in their ability to resist *in vivo* and *in vitro* oxidation of lipids. Study group included 15 women on oestradiol valerate (mean age 56 years, treatment duration 10.5 years) and 15 women on combined HRT with oestradiol valerate and levonorgestrel (mean age 58 years, treatment duration 11.3 years). In addition to lipid and apolipoprotein concentrations, the lagtime of LDL to oxidation, the rate of the propagation phase and the maximum concentration of conjugated dienes were recorded as indices of LDL susceptibility to copper-induced oxidation *in vitro*. As an *in vivo* marker of oxidative stress we measured 24-h excretion of urinary 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$). All measurements were done after long-term HRT (baseline), after 4 weeks pause and again 3 weeks after reintroduction of HRT. High-density lipoprotein (HDL) cholesterol and apolipoprotein AI concentrations were significantly higher and LDL to HDL ratio significantly lower after long-term oestradiol valerate therapy than after combined therapy. Simultaneously, the triglyceride and lipoprotein (a) levels were higher in the estrogen group. Susceptibility of LDL to oxidation and the level of 8-iso-$PGF_{2\alpha}$ were similar in both groups at all measurement points, and treatment group was not a statistically significant determinant of these markers at baseline. According to these results, estrogen and combined HRT do not differ in their abilities to oppose LDL oxidation *in vitro* or systemic oxidative stress *in vivo*, but have differential effects on blood lipids.

*Keywords*: Hormone replacement therapy; 8-iso-$PGF_{2\alpha}$; LDL oxidation; Lipoproteins; Oxidative stress

## INTRODUCTION

The major fraction of plasma cholesterol occurs in low-density lipoproteins (LDL) and elevated plasma level of LDL cholesterol is related to the development of atherosclerosis and consequently ischemic heart disease.[1] In atherosclerosis, LDL particles accumulate into the arterial wall intima as a result of modifications of which the most important is considered to be oxidation.[2,3] Oxidative modifications of LDL increase its uptake by macrophages that turn into lipid-laden foam cells.[4] Foam cell-formation is important in the development of the earliest visible signs of atherosclerosis, fatty streak lesions, which serve as precursors of advanced atherosclerotic plaques that can limit the blood flow and lead to clinical manifestations.[5]

Plasma concentration of LDL cholesterol increases after menopause,[6] rendering women more susceptible to coronary heart disease (CHD).[7] Postmenopausal hormone replacement therapy (HRT) with estrogen exerts favorable effects on lipid metabolism by reducing the concentration of LDL cholesterol and by increasing the high-density lipoprotein (HDL) cholesterol level.[8] The preparations with androgenic properties, or combined therapies containing androgenic progestogens, in turn, may have no effect on, or even decrease HDL cholesterol concentration. Despite the negative effects on HDL cholesterol level, combination therapy may have beneficial effects on some aspects of the lipoprotein

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2004 Taylor & Francis Ltd
DOI: 10.1080/10715760310001641169

profile; Levels of atherogenic lipoprotein (a), i.e. Lp(a), are markedly reduced by progestogens, while oestrogen has only a minimal effect.[9,10]

Considerable epidemiological evidence implies that oestrogen therapy substantially reduces the risk of morbidity and mortality from CHD,[11,12] which is in line with the observed beneficial effects of oestrogen on serum lipids. In addition to the effects on lipoprotein levels, short-term oestrogen use at supraphysiological level and a single large-dose of estrogen have been suggested to protect against coronary atherosclerosis by inhibiting the oxidation of LDL.[13–16] On the contrary, recent randomized controlled trials on women using combined hormone preparations have not shown the benefit of HRT for the risk of atherosclerosis and cardiovascular end points.[17–19] Consistent with these trials, some studies have found that oxidation of LDL particles is not influenced by HRT, given either in unopposed or combined preparations.[20–22] Contradictory results suggest that oestrogens' benefits on oxidation may be preserved or negated with concurrent progestin use and may substantially depend on the progestin preparation.

Evidence of proposed antioxidant effects of various oestrogens have primarily been obtained from *in vitro* experiments and limited evidence is available to support their antioxidative role *in vivo*. The aim of the present study was to clarify the effects of long-term HRT on the susceptibility of LDL to copper-induced oxidation in women on oestradiol valerate and on women on treatment combination of oestradiol valerate and levonorgestrel. Since copper-induced oxidation of LDL is an *in vitro* index of oxidation, we wanted also to see whether the systemic oxidative stress is different during the use of these two hormone preparations. Therefore, urinary 8-iso-prostaglandin $F_{2\alpha}$ i.e. (8-iso-$PGF_{2\alpha}$) level that reflects the free radical generation *in vivo*[23,24] was also measured.

## SUBJECTS AND METHODS

### Subjects

The study population consisted of 30 healthy, normotensive, non-smoking postmenopausal women. Of them 15 were hysterectomized and their HRT consisted of oestradiol valerate 2 mg/day. Mean age of these women was 56.4 ± 7.0 years and treatment duration 10.5 ± 3.2 years. For the other 15 women, the hormone regimen was oestradiol valerate 2 mg/day for 11 days followed by oestradiol valerate 2 mg/day + levonorgestrel 0.25 mg/day for 10 days. These women were 58.0 ± 4.1 years old and duration of their treatment was 11.3 ± 3.6 years. The mean body-mass index (BMI) was 24.6 ± 2.5 kg/m$^2$ in women on oestradiol valerate therapy and 26.2 ± 3.5 kg/m$^2$ in women on combined HRT. The women did not have diagnosed diabetes or cardiovascular diseases. The study was approved by the Ethical Committee of the Tampere University Hospital and subjects gave written informed consent.

### Lipid and Apolipoprotein Concentrations

All biochemical measurements were done at baseline after long-term HRT (time point 1) and were repeated 4 weeks after the treatment was discontinued (time point 2) and again 3 weeks after reintroduction of HRT (time point 3). Venous blood samples were drawn after a 12-h overnight fast and from women on combination therapy during the third week of the treatment cycle. Serum total cholesterol and triglycerides were measured by the dry slide technique (Ektachem 700 analyzer, Johnson and Johnson Clinical Diagnostics, Rochester, NY, USA). The HDL cholesterol concentration was determined after precipitation of LDL and very low-density lipoprotein with dextran sulfate/magnesium chloride[25] using the same technique. The LDL cholesterol concentration was calculated by Friedewald's formula.[26] Apolipoproteins AI and B were analyzed by using immunonephelometric method (Behring, Behringwerke AG, Marburg, Germany) and Lp(a) by using two-site immunoradiometric assay (Pharmacia, Uppsala, Sweden).

### Indices of Oxidation

Susceptibility of LDL to copper-induced oxidation was measured by a previously described method.[27] In brief, LDL was first isolated by ultracentrifugation at 100,000 rpm for 30 min (Beckman TLV-100, Palo Alto, USA) after which the purity of the preparation was verified by a lipoprotein agarose-gel electrophoresis with Sudan-Black lipid staining and the LDL protein concentration measured. Isolated LDL was oxidized in the presence of 1.65 μmol/l $CuSO_4$ and the production of conjugated dienes (i.e. hydroperoxides with conjugated double bonds) was monitored as changes in absorbance at 234 nm at 37 °C (Lambda Bio 10 spectrophotometer, Perkin Elmer, Überlingen, Germany). The lagtime of LDL to oxidation (min), the rate of the propagation phase (μmol/l of dienes/min) and the maximum concentration of conjugated dienes (μmol/g of LDL protein) formed were recorded during the oxidation period.

For the measurement of 8-iso-$PGF_{2\alpha}$, the total volume of 24-h urine was mixed and an aliquot was stored frozen at −70°C until analyzed. Thawed urine samples were centrifuged at 10,000*g* for 10 min, and, after proper dilution, the supernatant was used for the determination of 8-iso-$PGF_{2\alpha}$ by a competitive ELISA according to manufacturer's instructions (R & D Systems Inc., Minneapolis, USA) including

the quality control. Samples were analyzed in duplicates and the mean values were then used in the statistical analysis. Urinary 8-iso-$PGF_{2\alpha}$ was expressed as the total amount excreted in 24 h (ng/24-h).

### Statistics

Means of continuous variables between treatment groups were compared using *t*-test for independent samples or one-way analysis of covariance (ANCOVA) with the HDL cholesterol concentration, triglyceride level or the measurement series of the sample during the LDL oxidation experiment as a covariate. Statistical analyzes of the longitudinal data were carried out using analysis of variance for repeated measures (RANOVA) to establish any treatment period-by-treatment group interaction. In case of a significant interaction, the LSD post-hoc test was used after RANOVA to find out the pairwise differences. Pearson's correlation coefficient was used to evaluate correlations. In searching for the set of variables that could predict the lagtime to oxidation or 8-iso-$PGF_{2\alpha}$ level at baseline, we used multiple forward stepwise regression model with the following explanatory variables: treatment group, age, BMI, measurement series of the sample (in a case of the lagtime), apo AI, total cholesterol, LDL cholesterol, triglyceride, and Lp(a) level. Non-normally distributed data were analyzed after logarithmic transformation. Values are expressed as mean ± SD unless otherwise stated. A *p*-value of less than 0.05 was considered statistically significant. Statistica for Windows version 5.1 software package (Statsoft Inc., Tulsa, USA) was used for statistical analyzes.

## RESULTS

### Lipids and Apolipoproteins

Table I shows the serum lipid and apolipoprotein levels at baseline, after the break in the therapy and after reintroduction of therapy. Subjects who had used only oestradiol valerate had higher concentrations of HDL cholesterol and its major apolipoprotein, apo AI, at baseline and after the therapy was continued than subjects on combined therapy. At these two measurement points also the LDL/HDL ratio was lower in the oestradiol group compared to the combined therapy group. There was a statistically significant interaction between treatment groups and treatment periods considering HDL cholesterol, apo AI and LDL to HDL ratio ($p < 0.001$). In the oestradiol valerate group, the HDL cholesterol concentration remained stable over all study periods. Reintroduction of therapy after withdrawal, in turn, increased the apo AI concentration of these subjects by 11.5% ($p < 0.001$). In the combination therapy group, HDL cholesterol increased by 25.0% ($p < 0.001$) and apo AI concentration by 19.3% ($p < 0.001$) after the therapy was discontinued. These concentrations decreased again by 12.2% ($p < 0.05$) and by 9.8% ($p < 0.001$), when HRT was reintroduced after the break.

Concentration of triglycerides was not statistically significantly higher in estrogen therapy group in comparison with the combined therapy group in any of the studied time points, although the interaction between treatment groups and periods was statistically significant ($p = 0.026$). Among subjects on combination therapy, the triglyceride concentration increased when the therapy was on break, while among subjects on estrogen therapy the level remained stable. Moreover, the Lp(a) level was statistically significantly lower in subjects on combination therapy than in subjects on estrogen therapy during the break in therapy and after therapy was again continued. In both groups, the level tended to increase when therapy was discontinued ($p = 0.084$ for interaction).

### Susceptibility of LDL to Oxidation *In vitro*

Table II shows the lagtime of LDL to oxidation, oxidation rate and maximum diene concentration in

TABLE I Serum lipid levels in women on oestradiol valerate therapy and on treatment combination of oestradiol valerate and levonorgestrel after 10 years of therapy (baseline), after 4 weeks break in therapy and 3 weeks after reintroduction of therapy

| | Estrogen therapy (*n*=15) | | | Combined therapy (*n*=15) | | | Interaction† |
|---|---|---|---|---|---|---|---|
| | Baseline | Break | Continued | Baseline | Break | Continued | *p*-value |
| Total cholesterol (mmol/l) | 5.93 ± 1.16 | 6.19 ± 0.90 | 5.67 ± 0.83 | 5.65 ± 1.01 | 6.18 ± 1.12 | 5.61 ± 1.14 | 0.589 |
| LDL cholesterol (mmol/l) | 3.55 ± 1.14 | 3.87 ± 0.93 | 3.20 ± 0.88 | 3.98 ± 0.98 | 4.11 ± 0.86 | 3.81 ± 1.09 | 0.334 |
| HDL cholesterol (mmol/l) | 1.77 ± 0.42*** | 1.72 ± 0.59 | 1.85 ± 0.51** | 1.23 ± 0.28 | 1.55 ± 0.28 | 1.36 ± 0.32 | <0.001 |
| LDL/HDL ratio | 2.17 ± 0.93** | 2.55 ± 1.07 | 1.90 ± 0.81** | 3.38 ± 1.17 | 2.74 ± 0.73 | 2.99 ± 1.22 | <0.001 |
| Triglycerides (mmol/l) | 1.34 ± 0.68 | 1.32 ± 0.73 | 1.36 ± 0.70 | 0.94 ± 0.37 | 1.26 ± 0.41 | 0.97 ± 0.35 | 0.026 |
| Apolipoprotein AI (g/l) | 1.90 ± 0.26*** | 1.82 ± 0.31 | 2.03 ± 0.34*** | 1.45 ± 0.17 | 1.73 ± 0.20 | 1.56 ± 0.23 | <0.001 |
| Apolipoprotein B (g/l) | 0.91 ± 0.32 | 1.07 ± 0.35 | 0.94 ± 0.30 | 0.96 ± 0.25 | 1.02 ± 0.27 | 0.97 ± 0.26 | 0.110 |
| Lp(a) (g/l)‡ | 257 ± 395 | 355 ± 574* | 274 ± 428* | 81 ± 83 | 103 ± 117 | 87 ± 101 | 0.084 |

Between estrogen and combined therapy groups: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, *p*-values by *t*-test. † Interaction between treatment groups and treatment periods by RANOVA. ‡ Data were transformed logarithmically before analysis.

TABLE II Susceptibility of LDL to oxidation and urinary 8-iso-$PGF_{2\alpha}$ levels in women on oestradiol valerate therapy and on treatment combination of oestradiol valerate and levonorgestrel after 10 years of therapy (baseline), after 4 weeks break in therapy and 3 weeks after reintroduction of therapy

| | Lagtime to oxidation (min) | *p*-Value* | Oxidation rate (μmol/l/min) | *p*-Value* | Maximum diene concentration (μmol/g) | *p*-Value* | 8-iso-$PGF_{2\alpha}$ (ng/24-h) | *p*-Value† |
|---|---|---|---|---|---|---|---|---|
| Baseline | | | | | | | | |
| Estrogen therapy | 46.6 ± 8.3 | 0.149 | 0.69 ± 0.09 | 0.098 | 480 ± 56 | 0.123 | 2158 ± 2377 | 0.199 |
| Combined therapy | 51.7 ± 4.7 | | 0.78 ± 0.14 | | 529 ± 79 | | 3844 ± 3237 | |
| Break in HRT | | | | | | | | |
| Estrogen therapy | 46.5 ± 7.3 | 0.252 | 0.70 ± 0.11 | 0.226 | 480 ± 71 | 0.031 | 1909 ± 1959 | 0.426 |
| Combined therapy | 49.8 ± 5.1 | | 0.78 ± 0.11 | | 545 ± 64 | | 2599 ± 2663 | |
| Reintroduction of HRT | | | | | | | | |
| Estrogen therapy | 43.6 ± 8.5 | 0.173 | 0.66 ± 0.14 | 0.230 | 451 ± 92 | 0.055 | 2187 ± 2395 | 0.878 |
| Combined therapy | 49.9 ± 3.8 | | 0.76 ± 0.10 | | 536 ± 59 | | 1803 ± 1158 | |

*$p$-Values between groups by ANCOVA with the measurement series of the sample as a covariate. †Data were transformed logarithmically before analysis.

the two treatment groups at different time points. Considering lagtime, the estrogen and combined therapy groups did not differ at any time points, although subjects on combined therapy tended to have longer lagtime to oxidation compared to subjects on estrogen therapy. The oxidation rate tended to be higher in combined therapy group than in estrogen therapy group at baseline ($p = 0.098$). The maximum diene concentration was higher in subjects on combination therapy during the treatment withdrawal ($p = 0.031$) and tended to be higher after the therapy was reintroduced ($p = 0.055$). The results were similar when HDL cholesterol concentration or triglyceride level was used as a covariate in ANCOVA in addition to the measurement series of the sample. Figure 1 shows the lagtime and maximum diene concentration of these two treatment groups at different time points. There was no statistically significant interaction between treatment groups and treatment periods on the lagtime ($p = 0.334$, Fig. 1A) or the maximum diene concentration ($p = 0.314$, Fig. 1B).

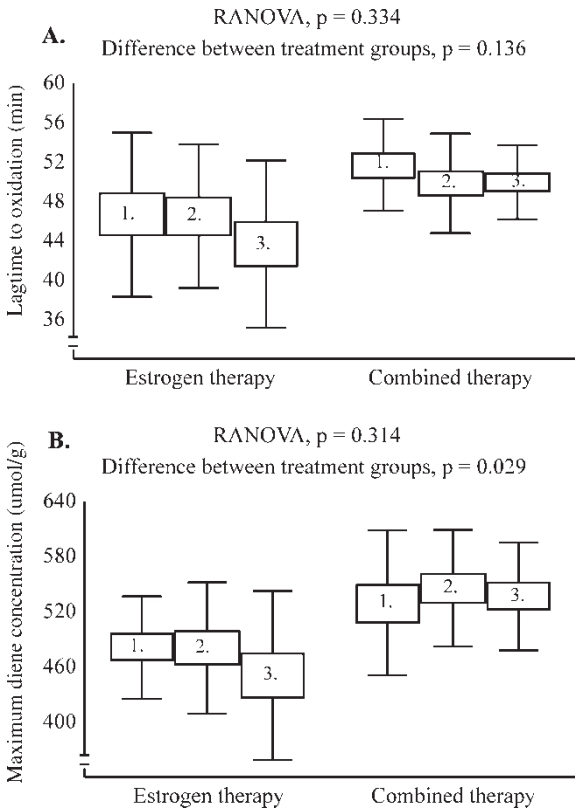


FIGURE 1 Lagtime of LDL to oxidation (Panel A) and maximum diene concentration (Panel B) after long term HRT (1.), 4 weeks after cessation of treatment (2.) and 3 weeks after reintroduction of therapy (3.). Interaction between treatment periods and groups are from analysis of variance for repeated measures (RANOVA) with the measurement series of the sample as a covariate. Standard errors are shown as boxes and standard deviations as whiskers.

### 8-iso-$PGF_{2\alpha}$ as a Marker of *In vivo* Oxidation

According to Table II, the 24-h urinary 8-iso-$PGF_{2\alpha}$ excretion was not statistically significantly different at baseline in women on estrogen therapy compared with women on combination therapy, although the excretion was moderately lower after long-term estrogen use ($p = 0.199$). The difference remained statistically non-significant when HDL cholesterol or triglyceride level was used as a covariate in ANCOVA. During the therapy break and after its reintroduction, the groups showed similar excretion level of 8-iso-$PGF_{2\alpha}$. The interaction between treatment groups and treatment periods was also statistically non-significant ($p = 0.444$).

### Determinants of Lagtime of LDL to Oxidation and Urinary 8-iso-$PGF_{2\alpha}$ Level at Baseline

Correlations between lagtime of LDL to oxidation and serum lipids and urinary 8-iso-$PGF_{2\alpha}$ are shown in Table III. In all women despite their treatment group, lagtime correlated negatively with apo AI, HDL cholesterol and triglyceride level. During the break in therapy, this correlation was lost. In subjects on oestradiol valerate treatment, the correlation with lagtime and apo AI remained at time point 3 and in combination therapy group at time point 1. In the combination therapy group, there was also a statistically significant correlation between HDL cholesterol concentration and lagtime at baseline. Considering 8-iso-$PGF_{2\alpha}$ and lagtime, women on estrogen therapy showed significant positive correlation after the therapy was restarted after the break. On the contrary, correlation between lagtime and 8-iso-$PGF_{2\alpha}$ level at baseline was negative in combined therapy subjects.

Multiple forward stepwise regression analysis was then performed to estimate the effect of treatment group and plasma lipid levels on lagtime and 8-iso-$PGF_{2\alpha}$ level at baseline (Table IV). The models were able to account for 24% of the variance of both the lagtime to oxidation and the 8-iso-$PGF_{2\alpha}$ level. The only statistically significant determinant of lagtime at baseline was the apo AI level. Triglyceride concentration and BMI were, in turn, the significant predictors of 24-h excretion of 8-iso-$PGF_{2\alpha}$. Treatment group was dropped from both models.

## DISCUSSION

Over the past few decades, epidemiological studies have indicated that postmenopausal HRT (with most using conjugated equine estrogens) can reduce mortality from CHD and other cardiovascular diseases by approximately 50%.[12] Contrary to these observational studies, the Heart and Estrogen/Progestin Replacement Study (HERS) demonstrated that combined therapy with conjugated equine estrogen plus medroxyprogesterone acetate (MPA) does not reduce the overall rate of coronary events, progression of angiographically defined coronary atherosclerosis, or incidence of cerebrovascular events in postmenopausal women.[18,19,28] This suggests that the beneficial effects of estrogen could be partly offset by concomitant harmful effects and that concurrent

TABLE III Correlations between lagtime and lipids, apolipoproteins and urinary 8-iso-$PGF_{2\alpha}$ in women with oestradiol valerate therapy and in women with treatment combination of oestradiol valerate and levonorgestrel after 10 years of therapy (baseline), 4 weeks after the treatment was discontinued and 3 weeks after its reintroduction

| | Lagtime of LDL to oxidation (min) | | |
|---|---|---|---|
| Pearson's correlation coefficient | Estrogen ($n$=15) $R$ | Combined ($n$=15) $R$ | All subjects ($n$=30) $R$ |
| Apolipoprotein AI (g/l) | | | |
| Baseline | −0.167 | −0.639* | −0.448** |
| Break in HRT | 0.055 | −0.240 | −0.080 |
| Reintroduction of HRT | −0.700** | −0.217 | −0.697*** |
| HDL cholesterol (mmol/l) | | | |
| Baseline | 0.013 | −0.535* | −0.318 |
| Break in HRT | 0.068 | −0.034 | −0.007 |
| Reintroduction of HRT | −0.442 | −0.125 | −0.522** |
| Triglyceride (mmol/l) | | | |
| Baseline | −0.360 | 0.150 | −0.342 |
| Break in HRT | −0.146 | 0.008 | −0.117 |
| Reintroduction of HRT | −0.437 | 0.206 | −0.430* |
| 8-iso-$PGF_{2\alpha}$ (ng/24-h)† | | | |
| Baseline | 0.367 | −0.529* | 0.117 |
| Break in HRT | 0.416 | 0.105 | 0.320 |
| Reintroduction of HRT | 0.550* | −0.195 | 0.330 |

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. †Data were transformed logarithmically before analysis.

TABLE IV Regression model of factors predicting lagtime of LDL to oxidation and urinary 8-iso-$PGF_{2\alpha}$ at baseline in all women ($n = 30$)

| | Standardized regression coefficient | *p*-Value |
|---|---|---|
| Determinants of lagtime (min) | | |
| Apolipoprotein AI (g/l) | −0.409 | 0.018 |
| Total cholesterol (mmol/l) | −0.308 | 0.069 |
| Total model: $R^2$=24.3%, $p$=0.009 | | |
| Determinants of 8-iso-$PGF_{2\alpha}$ (ng/24-h)* | | |
| Triglycerides (mmol/l) | −0.486 | 0.012 |
| Body-mass index (kg/$m^2$) | 0.486 | 0.012 |
| Lipoprotein (a) (g/l)* | 0.236 | 0.169 |
| Total model: $R^2$=24.3%, $p$=0.016 | | |

Multiple forward stepwise regression model with treatment group, age, BMI, measurement series of the sample (in a case of lagtime), apo AI, total cholesterol, LDL cholesterol, triglycerides and Lp(a) as explanatory variables. * Data were transformed logarithmically before analysis.



progesterone therapy may oppose some beneficial effects of estrogens on biological systems, such as lipoprotein and carbohydrate metabolisms and the clotting cascade,[29] and further on cardiovascular health. Since it has been suggested that estrogens may be potent antioxidants, we asked if combined HRT could affect also the antioxidant protection coupled with estrogen. As a result, we found no significant differences in the ability of LDL to resist oxidation *in vitro* between women on long-term estrogen HRT and women on combined HRT. Also, the excretion of *in vivo* marker of systemic oxidative stress, 8-iso-$PGF_{2\alpha}$, was similar in these two HRT groups. The changes in lipoprotein levels were evident and identical to many previously published studies.

It is well documented that beneficial effects of estrogen include decreased LDL cholesterol and increased HDL cholesterol concentrations.[8] The decrease in LDL cholesterol level is due to increased hepatic synthesis of LDL receptors and increase in HDL cholesterol level to estrogen-induced inhibition of hepatic lipase.[30] The preparations with androgenic properties, or combined therapies containing androgenic progestogens, may, however, decrease HDL cholesterol level. This was seen also in the present study. In the combination therapy group the HDL cholesterol concentration increased statistically significantly when the therapy was discontinued. Despite the negative effects on HDL cholesterol level, combination therapy may have some beneficial effects on the lipoprotein profile. Levels of Lp(a), which is similar in composition to LDL and considered particularly atherogenic, are markedly reduced by progestogens, while oestrogen has only a minimal effect.[9,10] As expected, this was seen also in our two study groups. Moreover, the ability of estrogen to increase the level of triglycerides was observed, which could, in fact, increase the risk of cardiac events in postmenopausal women with established coronary disease.[31] Despite the effects of HRT on serum lipid levels, HRT seems not to reduce the CHD risk in either primary or secondary prevention situations according to randomized, controlled clinical end-point trials (for review, see Ref. [32]). The present study did not analyze the overall CHD risk associated with HRT.

The oxidative modification of LDL plays a key role in the development of atherosclerosis.[2] Lp(a) can be modified by oxidation in similar fashion as LDL and therefore it can precipitate the atherosclerotic lesion formation.[33] Biological oxidative modification can be mimicked simply by incubating LDL in the presence of copper ions. Although incubation of LDL with copper is unphysiological, a number of studies have used this method to estimate *in vivo* situation. Reports of an antioxidant effect of oestrogen have, however, remained contradictory. Short-term studies have reported that treatment with transdermal oestradiol significantly increased,[16] moderately increased[22] or had no effect[20] on the lagtime to copper-mediated oxidation of LDL. One short-term study reported that oral estrogen reduced the susceptibility of LDL to oxidation,[34] which conflicts with the results from two studies that did not find any antioxidant effect of oestrogen given orally.[20,21] The information on the action of progestins on LDL oxidation is insufficient. While some experimental[35,36] and clinical[20] studies have shown neutral effects, some investigators have found that high dose progestins and particularly MPA, oppose the antioxidant action of estrogens.[35,37]

The results of our study are not fully comparable with the results of previous studies since the progestin preparation of the combination therapy regimen is different. In our study, the lagtime to copper-induced oxidation was similar in women using estrogen only and in women using combined HRT. One month break in therapy did not affect lagtime suggesting that HRT is not its major determinant. Also according to the regression analysis, only the apo AI concentration predicted lagtime at baseline whereas the study group did not. In addition, apo AI and HDL cholesterol level and triglyceride concentration correlated negatively with lagtime. It is quite unexpected that apo AI

concentration explains lagtime to oxidation. However, one has to bear in mind that oxidation can partially take place already in circulation, which could affect lagtime to oxidation *in vitro*; Apo AI is not present in the LDL fraction that is oxidized by copper ions and its probable effects have to have happened already earlier. It is therefore likely that apo AI is not directly affecting oxidative susceptibility of LDL but rather reflecting some events in lipid metabolism *in vivo*.

Also in the light of previous studies, it seems to be obscure whether HRT can affect oxidation by a direct mechanism: First, LDL particles contain many natural antioxidants able to trap free radicals. Lagtime to oxidation is thus intimately linked especially to the levels of α-tocopherol and ubiquinone.[38] The concentrations of antioxidant were not measured in the present study, although there is no previous evidence that HRT would affect vitamin E or ascorbic acid status.[39,40] Second, the estrogen-induced increase in plasma triglyceride concentration has been shown to affect LDL particle size,[41] resulting in an increased proportion of small dense LDL particles, which are easily oxidized. On the other hand, the increased level of HDL cholesterol might retard LDL oxidation during estrogen HRT.[42] Combined HRT, in turn, is proven to decrease triglyceride level and consequently the level of small LDL particles, but simultaneously also the HDL cholesterol concentration. In both cases opposing antioxidative and oxidative effects might result in no net change in the susceptibility to oxidation. The borderline difference between groups in the maximum diene concentration is also likely to be due to changes in plasma lipoprotein and fatty acid composition during HRT, since maximum diene concentration reflects the amount of polyunsaturated fatty acids in LDL;[38] it is not a direct index of oxidation. Our study thus suggests that while HRT seems not to directly affect the susceptibility of LDL to copper-induced oxidation, the HRT-induced changes in the composition of lipoproteins perhaps do.

Despite antioxidant effects being exhibited by various oestrogens and their metabolites, limited evidence is available to support their antioxidant role *in vivo*. The one study that has focused on *in vivo* oxidation of lipids after the long-term HRT showed that there were equal amounts of antibodies against oxidized LDL in subjects on estradiol valerate alone, combined estradiol valerate and levonorgestrel, and on combined estradiol valerate and MPA.[43] We measured the excretion of 8-iso-$PGF_{2\alpha}$ in the urine, since it is supposed to be a useful non-invasive *in vivo* index of free radical generation. The levels of $F_2$-isoprostanes increase dramatically in smokers,[24] experimental animals subject to oxidant injury,[44] and in patients with acute myocardial infarction.[45] In addition, increased 8-iso-$PGF_{2\alpha}$ level has been reported after a diet containing high amounts of polyunsaturated fatty acids[46] and after intake of conjugated linoleic acid,[47] that are prone to oxidation. In our study, the level of 8-iso-$PGF_{2\alpha}$ in the urine was moderately decreased after long-term oestradiol valerate therapy compared with combined therapy. However, therapy group was not accepted in the regression model as an explanatory variable for the 8-iso-$PGF_{2\alpha}$ level at baseline unlike triglyceride level and BMI, which indicates that there are no differences between groups in this respect. Still, the size of our study population was relatively small and therefore it is possible that we were unable to detect minor differences in the indices of oxidation. During the treatment break, there, however, were clear changes in the lipid and apolipoprotein levels. It is thus likely that 4 weeks withdrawal period would also be enough to detect changes in the oxidation parameters if they would have existed. In one short-term study, plasma levels of free 8-iso-$PGF_{2\alpha}$ decreased significantly after 6 weeks of treatment combination of conjugated equine estrogen and MPA in women with high incidence of obesity, smoking and diabetes.[48] Whether the conflicting results between this study and the present study are due to different hormonal preparations, cardiovascular risk factors among subjects or time frames is not clear at the present moment.

When comparing oxidation markers of the present study, there was no significant correlation between the lagtime to oxidation and the 8-iso-$PGF_{2\alpha}$ level and, according to the regression analysis, the statistically significant determinants of these factors at baseline were different. Therefore, our results imply that these two markers are representatives of different oxidation events and question, whether the lagtime to oxidation is really a good index to estimate *in vivo* situation of oxidative stress.

Our study supports the evidence that oestrogen valerate therapy, but not combined therapy, increases the concentration of HDL cholesterol. However, this treatment also increases plasma triglyceride and Lp(a) level, which may add the risk of CHD. In addition to the effects on plasma lipid profile, it has been suggested that HRT with estrogen, but not combined therapy with estrogen and progestin, may have antioxidative effects. In our study, the urinary excretion of an *in vivo* oxidative stress marker (8-iso-$PGF_2$) and the *in vitro* index of LDL susceptibility to oxidation (lagtime) were not statistically significantly different between oestradiol valerate users and oestradiol valerate and levonorgestrel users, suggesting that these two hormone therapies do not differ in their abilities to resist oxidative stress.

*Acknowledgements*

The study was supported by the Medical Research Fund of Tampere University Hospital, the Finnish Foundation for Cardiovascular Research, and the Research Foundation of Orion Corporation. The authors thank Marita Koli for skilful laboratory assistance.

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