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Markers of inflammation in women on different hormone replacement therapies

Margit Fröhlich[1,2], Nikolai Mühlberger[3], Hartmut Hanke[1], Armin Imhof[1], Angela Döring[4], Mark B Pepys[5] and Wolfgang Koenig[1]

BACKGROUND AND AIM. To measure inflammatory markers in postmenopausal women on different forms of hormone replacement therapy (HRT).

METHOD. C-reactive protein (CRP), fibrinogen, plasma viscosity (PV), albumin and white blood cell (WBC) count were determined in 749 postmenopausal women.

RESULTS. CRP concentration was significantly higher in women on estrogen monotherapy (difference of the median (d) 0.96 mg/l, $P = 0.013$), compared to those without HRT, but there was no difference in women on combined HRT. Fibrinogen concentration was significantly lower in women on estrogen monotherapy (d 0.25 g/l, $P = 0.004$) and combined HRT (d 0.4 g/l, $P < 0.001$), compared to women without HRT. Similarly, PV was significantly lower in women on estrogen monotherapy (d 0.017 mPa·s, $P = 0.007$) and women on combined HRT (d 0.039 mPa·s, $P < 0.001$), compared to those without HRT. No differences were found for WBC count and the negative acute phase marker albumin in the various treatment groups. In contrast to oral estrogen administration, levels of CRP, fibrinogen and PV in women on transdermal estrogen therapy did not differ from the no-HRT group. There was no association between these markers of inflammation and plasma estrogen levels.

CONCLUSION. Oral estrogen monotherapy was associated with highest concentrations of CRP. In contrast, other markers of inflammation were either similar or lower in the oral HRT group, compared to the group of women without HRT, suggesting that higher CRP concentrations reflect estrogen effects on CRP expression rather than a systemic pro-inflammatory effect.

Keywords: coronary disease; C-reactive protein; fibrinogen; hormone replacement therapy; inflammation; plasma viscosity

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Introduction

Cardiovascular morbidity and mortality in women increase significantly after menopause (1), reflecting at least in part the altered hormonal balance. Although observational studies indicate a reduced coronary heart disease (CHD) risk in women on HRT (reviewed in (2)), recent randomized prospective studies (Heart and Estrogen/Progestin Replacement Study (HERS) (3), Estrogen Replacement and Atherosclerosis (ERA) trial (4), Women's Health Initiative (WHI) Study (5)), showed no beneficial effects. Furthermore, there are reports of an increased risk of thromboembolic events in HRT users (6–8).

Atherosclerosis and thrombosis are closely related (9) and there is evidence for a thrombogenic state in patients at increased risk for acute ischemic events (10, 11). Prothrombotic and antifibrinolytic proteins such as fibrinogen (11, 12) and plasminogen activator inhibitor-1 (PAI-1) (13) have been identified as independent risk factors for CHD. Most of these proteins are part of the acute phase reaction and increase in response to inflammatory stimuli (14). Furthermore, CRP – a very sensitive acute phase protein – has been shown to predict the risk of future CHD (15–19), supporting the hypothesis that inflammatory processes contribute significantly to the pathogenesis of atherothrombotic disease (20, 21).

CRP values have lately been reported to be increased in women on hormone therapy (22–25). In

From the [1]Department of Internal Medicine II – Cardiology, University of Ulm Medical Center, Ulm, Germany, [2]Department of Internal Medicine VI – Clinical Pharmacology and Pharmacoepidemiology, University Hospital of Heidelberg, Heidelberg, Germany, [3]GSF – National Research Centre for Environment and Health, IGM Institute, Neuherberg, Germany, [4]GSF – National Research Centre for Environment and Health, Institute of Epidemiology, Neuherberg, Germany, [5]Department of Medicine, Royal Free and University College Medical School, London, UK.

Correspondence: Wolfgang Koenig, MD, Abteilung Innere Medizin II – Kardiologie, Medizinische Universitätsklinik, Robert-Koch-Straße 8, D-89081 Ulm, Germany. Fax: +49 731 500 33872: E-mail: wolfgang.koenig@ medizin.uni-ulm.de

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Abbreviations and acronyms

BMI	body mass index
CHD	coronary heart disease
CHOD-PAP	cholesterol oxidase-peroxidase amino-phenazone
CRP	C-reactive protein
EDTA	ethylenediamine tetraacetic acid
ERA	the Estrogen Replacement and Atherosclerosis trial
HDL	high density lipoprotein
HERS	the Heart and Estrogen/Progestin Replacement Study
HRT	hormone replacement therapy
IQ	inter quartile
LDL	low density lipoprotein
MONICA	the Monitoring of Trends and Determinants in Cardiovascular Disease study
PEPI	the Postmenopausal Estrogen/Progestin Interventions study
PV	plasma viscosity
PAI-1	plasminogen activator inhibitor-1
SAP	serum amyloid P
WBC	white blood cell
WHI	the Women's Health Initiative
WHS	Women's Health Study

Key messages

- **There is no systemic pro-inflammatory effect of HRT on markers of inflammation.**
- **In women on HRT, concentrations of CRP, fibrinogen and PV vary substantially with the addition of a progestin and route of administration.**

contrast, HRT is associated with a decrease in fibrinogen (26, 27) and PAI-1 (28) values, and with a reduction in acute phase coagulation proteins and PV (26, 29), indicating that estrogens do not have a global pro-inflammatory effect. However, the actions of estrogens vary with dose, whether the formulations are synthetic or natural, the route of administration is oral or transdermal, and the concurrent use of a progesterone (30). These factors were not adequately defined in many of the previous studies, but may be crucial with regard to the results of the HERS (3) and WHI (5) study in which an oral combined HRT (conjugated equine estrogens plus medroxyprogesterone acetate) was administered. We have therefore measured fibrinogen, PV and CRP in postmenopausal women from a large population-based sample and looked for potential differences between women on oral estrogen monotherapy compared to estrogen/progesterone combination therapy, as well as those on transdermal estrogens.

Methods

The MONICA (Monitoring of Trends and Determinants in Cardiovascular Disease) Project is a WHO-coordinated observational long-term study with the main aims of measuring trends in cardiovascular morbidity and mortality and assessing their relationship to changes in risk factor levels and medical care (31, 32). It comprises a coronary event register and three independent cross-sectional studies in 5-year intervals. The present analysis involved all 1026 women aged 45–64 years among those participating in the third MONICA Augsburg survey in 1994/95. Among those, 208 still took oral contraceptives, had their monthly period, or were pregnant. Eight hundred and eighteen women were menopausal (absence of the monthly period) or received HRT. Outcome parameters were missing for 69 women, thus, complete data were available from 749 women of whom 228 were HRT users. The most frequently prescribed preparations contained estradiol (40%), estradiolvalerate (32%), and conjugated estrogens (22%) as estrogenic component and levonorgestrel (40% of the combination therapies) and norethisteroneacetat (36% of the combination therapies) as progestagenic component. Fifty six per cent of the combination therapies were administered in a sequential fashion, however, blood collection was not standardized to a specific phase of HRT intake. After a standardized interview, during which HRT use was recorded in detail, blood pressure was measured according to the recommendations of the American Heart Association (33). Body height and weight were measured in light clothing, and body mass index (BMI) was computed as weight divided by the square of height (kg/m^2). Smoking behavior and alcohol consumption were determined as described elsewhere (34).

Non-fasting venous blood samples were drawn into ethylenediamine tetraacetic acid (EDTA) with the subjects in a supine resting position with only short-term venous occlusion and minimal suction. Red blood cell count, WBC count, hematocrit and platelet count were determined (Coulter Counter). EDTA blood was also immediately centrifuged at 3000 g for 15 min, and the plasma aliquoted and stored at −70°C. PV was measured at 37°C in a Coulter-Harkness capillary viscometer (Coulter Electronics, Luton, UK) (35). Fibrinogen and albumin concentrations were determined by immunonephelometry (Dade Behring, Marburg, Germany). CRP concentration was measured by an immunoradiometric assay (range 0.05–10 mg/l) calibrated with the WHO reference standard 85/506 (36). Total estradiol level

Table 1. Means and standard deviations of biochemical and anthropometrical variables in women taking and not taking HRT

Variables	HRT *n = 228*		No HRT *n = 521*		P[a]-*values*
Age (years)	54.1	(4.7)	56.6	(4.8)	<0.0001
Albumin (g/l)	42.2	(4.1)	42.7	(4.2)	0.0794
Leukocytes (10^9/l)	7.0	(1.8)	6.9	(1.7)	0.3396
BMI (kg/m^2)	26.6	(4.0)	28.5	(5.0)	0.0001
SBP (mmHg)	133.0	(20.0)	134.6	(19.6)	0.2978
DBP (mmHg)	81.0	(10.7)	81.9	(10.9)	0.2752
LDL-Chol. (mmol/l)	3.67	(0.97)	4.08	(1.06)	<0.0001
HDL-Chol. (mmol/l)	1.56	(0.44)	1.49	(0.42)	0.0265
Alcohol cons. (g/d)	8.9	(12.9)	6.2	(11.5)	0.0001
Education (years)	10.4	(2.1)	9.9	(1.9)	0.0001
Diabetes (N (%))	5	(2.2)	33	(6.3)	0.017
Smoking (N (%))					
– never	142	(62.3)	376	(72.2)	
– ex	49	(21.5)	72	(13.8)	
– occasional	6	(2.6)	7	(1.3)	
– regular	31	(13.6)	66	(12.7)	0.001
	n = 223		*n* = 59		
Estrogen (pg/mL)	125.3	(161.0)	78.1	(130.0)	0.0019

BMI = Body Mass Index; SBP = systolic blood pressure; DBP = diastolic blood pressure; LDL-Chol = LDL-cholesterol; HDL-Chol = HDL-cholesterol; Alcohol cons. = alcohol consumption.

[a]*P*-values = *P* for difference hormone replacement therapy *versus* no hormone replacement therapy

was determined by a radioimmunometric assay (Hermann Biermann GmbH, Bad Nauheim, Germany) with an assay range of 20–3600 pg/ml. Total and high density lipoprotein (HDL)-cholesterol were measured by routine enzymatic methods (cholesterol oxidase–peroxidase aminophenazone (CHOD-PAP) kit, Boehringer, Mannheim, Germany), and low density lipoprotein (LDL)-cholesterol was calculated according to Friedewald (37).

Routine laboratory tests, including blood cell counts, total cholesterol and HDL-cholesterol were subject to continuous internal and external quality control. Coefficients of variation for repeated measurements were 0.7% for PV, 5.0% for fibrinogen, 6.9% for albumin and 12% for CRP over all ranges.

Statistical methods

Homogeneity of the exposure groups was tested by Chi-square statistics, respectively Chi-square statistics for trend in case of categorical data, and Student t-tests, Wilcoxon sign rank tests or F-Test statistics in case of continuous data. Due to the skewed distribution of CRP values, differences in all outcome parameters (CRP, fibrinogen, PV) were analyzed either by non-parametric tests applied to crude values or parametric tests applied to log-transformed values. HRT effect on outcome parameters was additionally estimated in adjusted linear regression models. Stepwise backward elimination directed by Wald statistics to the 5% alpha level was the main confounder selection strategy. The following potential confounders were included in the full model: age, BMI, systolic and diastolic blood pressure, total cholesterol, LDL- and HDL-cholesterol, red blood cell count, WBC count, platelet count, hemoglobin, hematocrit, alcohol consumption, smoking, diabetes, education years, physical activity, history of angina (Rose questionnaire) and myocardial infarction, and finally lipid lowering and antihypertensive medication. In order to check the results' robustness to the applied confounder selection strategy, all analyses were repeated using a more liberal cutoff (alpha = 20%) and alternative test statistics (collapsibility test). Spearman rank correlation analyses, as well as adjusted linear regression analyses – as described above – were performed between estrogen levels and HRT and estrogen levels and all outcome parameters. We restricted these analyses to 59 randomly selected women without HRT and 223 women on HRT from which complete data on estrogen levels were available. Due to the low sensitivity of the estrogen assay, estrogen measurements of 108 women on HRT were below the detection limit of 20 pg/ml. All computations were performed with SAS software (SAS Windows 6.12). *P*-values <0.05 were considered statistically significant.

Results

Women on HRT were significantly younger, leaner, more educated, had significantly lower LDL-cholesterol levels, higher HDL-cholesterol levels, and had a lower incidence of diabetes compared to women

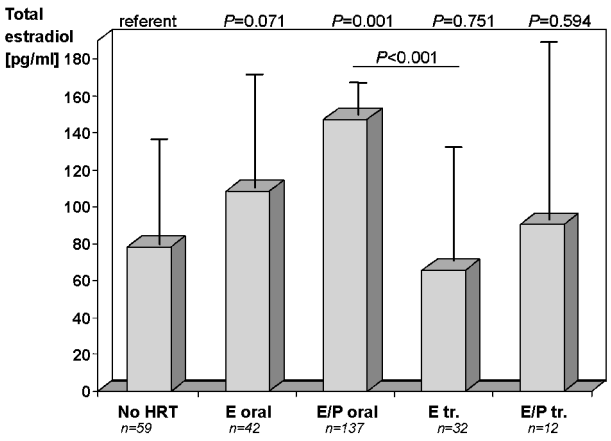

Figure 1. Mean, SD and unadjusted *P*-values of total estradiol for various forms of hormone replacement therapy *versus* no hormone replacement therapy E oral = oral estrogen monotherapy; E/P oral = oral combined hormone replacement therapy; E tr = transdermal estrogen monotherapy; E/P tr = transdermal combined hormone replacement therapy.

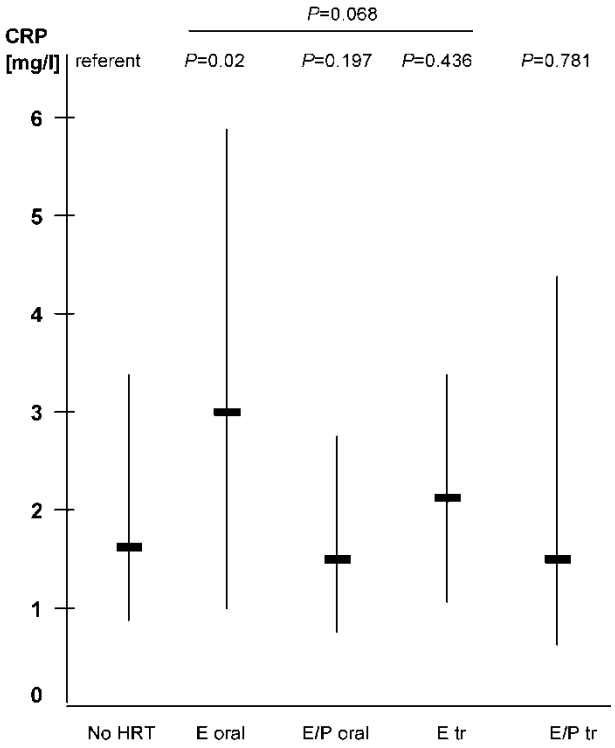

Figure 2

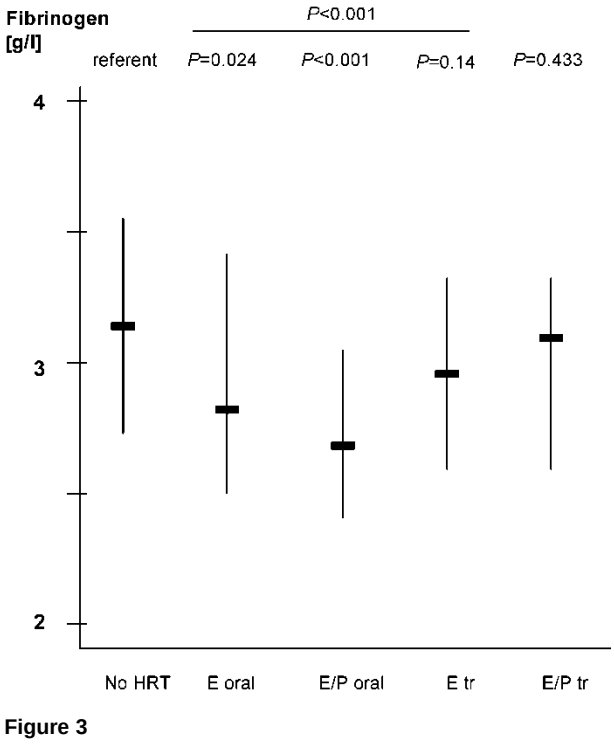

Figure 3

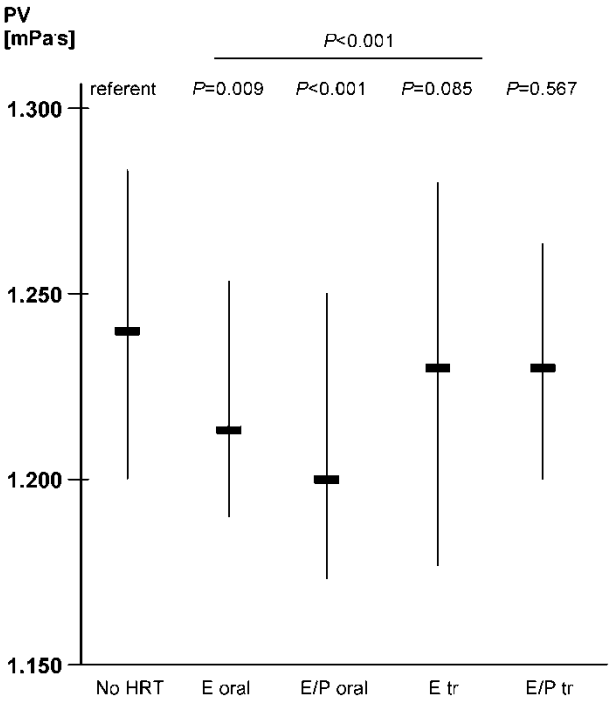

Figure 4

Figure 2–4. Distribution and unadjusted *P*-values of C-reactive protein, fibrinogen and plasma viscosity for various forms of hormone replacement therapy *versus* no hormone replacement therapy (referent). Also given is the *P*-value for the results of ANOVA. Graphics show medians and interquartile ranges for each variable in the different treatment groups. E oral = oral estrogen monotherapy; E/P oral = oral combined hormone replacement therapy; E tr = transdermal estrogen monotherapy; E/P tr = transdermal combined hormone replacement therapy.

Table 2. Median, interquartile (IQ)-range and *P*-values of CRP, PV and fibrinogen in women on estrogen monotherapy and estrogen/progesterone combination therapy compared to women without HRT.

Variable	No HRT n = 521		Estrogen monotherapy n = 77		Estrogen/progesterone combination n = 151	
CRP (mg/l)	1.62	(0.86–3.31)	2.58	(1.20–3.66)[a]	1.49	(0.77–2.85)[c]
Fibrinogen (g/l)	3.11	(0.73–3.55)	2.86	(2.53–3.32)[a]	2.71	(2.40–3.10)[bc]
PV (mPa·s)	1.241	(1.203–1.281)	1.224	(1.180–1.264)[a]	1.202	(1.173–1.254)[b]

[a]$P < 0.05$, [b]$P < 0.001$ = P for difference treatment *versus* no treatment.
[c]$P < 0.05$ = P for difference estrogen monotherapy *versus* estrogen/progesterone combination.

without HRT (Table 1). Leisure-time physical activity was significantly higher in women on HRT compared to those without HRT (data not shown), alcohol consumption was higher and there was a higher prevalence of smoking among women on HRT. There were no differences in blood pressure values, WBC count and albumin levels between the two groups. Estrogen levels were higher in women on HRT. However, on more detailed analyses this was only applicable to those women on combined oral therapy (Figure 1). Furthermore, no significant association could be shown between markers of inflammation and plasma estrogen levels (data not shown).

The median CRP value in women on estrogen monotherapy was significantly higher (difference 0.96 mg/l, $P = 0.013$) and the median fibrinogen (difference 0.25 g/l, $P = 0.004$) and PV (difference 0.017 mPa·s, $P = 0.007$) values were significantly lower compared to women without HRT (Table 2). CRP values in women on combined estrogen/progesterone therapy did not differ from those in untreated women, whereas fibrinogen and PV values were significantly lower in the combination group compared to women without HRT (difference 0.4 g/l, $P < 0.001$; 0.039 mPa·s, $P < 0.001$ for fibrinogen and PV, respectively). Furthermore, women on the combination therapy had significantly lower CRP and fibrinogen values compared to women on estrogen monotherapy (difference 1.09 mg/l, $P = 0.003$; 0.15 g/l, $P = 0.05$ for CRP and fibrinogen, respectively). These results did not change appreciably after adjusting for possible confounders mentioned in the methods section.

Figures 2–4 and Table 3 show the effect of various forms of HRT on CRP, fibrinogen and PV in unadjusted (Figs 1–3) and adjusted analyses (Table 3). Compared to the preceding analyses an additional discrimination was performed between women on oral and transdermal estrogen therapy. *P* values are shown for each form of HRT compared with the no HRT reference group. CRP values were significantly higher in women on oral estrogen monotherapy, but there was no difference between the other treatment groups and those not on HRT. In particular, there was no significant difference in women on transdermal therapy. Fibrinogen and PV values were significantly lower in women on combined oral estrogen/progesterone therapy, compared to the no-HRT reference group. A trend towards lower fibrinogen and PV values in women on oral or transdermal estrogen monotherapy did not reach statistical significance.

Discussion

In this population-based sample, women on HRT had significantly lower fibrinogen and PV levels than women without HRT. Conversely, CRP levels were significantly higher in women on oral estrogen monotherapy compared to those without HRT, even after adjustment for a variety of potential confounders. In more detailed analyses, comparing women on estrogen monotherapy with women on combined estrogen/progesterone therapy, CRP and fibrinogen levels were significantly lower in the combination group indicating a definite progesterone effect on these markers of inflammation and hemostasis. Transdermal estrogens were not associated with higher CRP levels, but differences between these groups were not statistically significant, possibly reflecting the small numbers of subjects in each group. As expected, estrogen levels were higher in women on HRT, however this was only due to women on combined oral therapy. Transdermal estrogen therapy was not associated with higher plasma estrogen levels. Furthermore, no association could be shown between CRP, PV, fibrinogen and estrogen levels.

Our results confirm previous reports of higher CRP values in postmenopausal women on HRT (22–24). This effect was mainly due to higher CRP concentrations in women on estrogen monotherapy (ΔCRP 0.96 mg/l), which is in the same order (ΔCRP 0.9 mg/l) as reported in the Postmenopausal Estrogen/Progestin Interventions (PEPI) trial (24) after 3 years of active treatment with oral conjugated equine estrogens. In contrast, women on combined estrogen/progesterone therapy showed no elevated CRP values in our study, whereas the PEPI trial reported an increase in CRP for various treatment assignments with combined HRT. Progesterones with pronounced

Table 3. Adjusted linear regression models estimating the effect of various forms of HRT *versus* no HRT on log CRP, log fibrinogen, and log plasma-viscosity.

Effect	Estimate	Lower 95% CL	Upper 95% CL	P-value
Log CRP:				
INTERCEPT	−4.097	−5.043	−3.150	0.0001
Estrogen monotherapy	0.700	0.419	0.982	0.0001
Estrogen/progesterone	0.123	−0.051	0.298	0.1648
Transd. estrogen/progesterone	0.165	−0.350	0.680	0.5289
Transd. estrogen	0.212	−0.108	0.532	0.1936
Log fibrinogen:				
INTERCEPT	−0.059	−0.230	0.181	0.6285
Estrogen monotherapy	−0.016	−0.076	0.045	0.6094
Estrogen/progesterone	−0.060	−0.098	−0.022	0.0021
Transd. estrogen/progesterone	−0.002	−0.113	0.109	0.9690
Transd. estrogen	−0.041	−0.110	0.028	0.2388
Log PV:				
INTERCEPT	−0.094	−0.155	−0.033	0.0025
Estrogen monotherapy	−0.006	−0.020	0.007	0.3636
Estrogen/progesterone	−0.013	−0.022	−0.005	0.0027
Transd. estrogen/progesterone	0.001	−0.024	0.026	0.9486
Transd. estrogen	−0.012	−0.028	0.004	0.1306

Model selection: *P*-Value directed backward elimination, alpha = 0.05 (see statistical methods section).
CL = confidence limit; transd. = transdermal.

androgenic properties (norgestrel, norethisterone), which were used most frequently in the present study population, may counteract the stimulation of hepatic protein production by estrogens (38). The predominant use of non-androgenic progesterones (medroxyprogesterone acetat, micronized progesterone) in the PEPI trial may thus account for the different findings.

Two studies (39, 40) have reported no significant changes in CRP concentrations of an isolated transdermal estrogen therapy, as we could show in the present study, and in the only study (41) of combined transdermal therapy, conducted in 33 postmenopausal women with type-2 diabetes, a significant decrease in CRP concentration was observed after 6 months of treatment (ΔCRP 0.5 mg/l). There were only 12 women on the same treatment regime in the present study and their CRP values did not differ from the no-HRT group.

Elevated levels of CRP in women on HRT, and in particular in those on oral estrogen monotherapy, may have significant clinical implications. Increased CRP levels are associated with an increased incidence of myocardial infarction, stroke, peripheral vascular disease (15–19), and with an adverse outcome in patients with unstable CHD (42). These associations have also been convincingly confirmed in the Women's Health Study (WHS) (43) in which baseline CRP levels in the top quartile were associated with an adjusted relative risk of 5.5 for myocardial infarction and stroke.

It is unclear at present, whether CRP is just a marker of inflammatory processes in the atherosclerotic artery wall or elsewhere in the body or whether it actively contributes to atherothrombosis. However, there are several plausible mechanisms by which CRP, which is generally present within atherosclerotic plaques (44, 45) may contribute to atherogenesis and/or thrombosis, including its capacity to bind LDL (46), to mediate foam-cell formation (47), to activate complement (48, 49), to decrease endothelial nitric oxide synthase (eNOS) expression (50), and to stimulate tissue factor production by macrophages (51).

The present data also confirm our own and other previous findings (25–29) of lower levels of both fibrinogen and PV in women on HRT compared to no HRT, and even lower levels in both parameters in women on combined HRT. Fibrinogen is a major determinant of PV (52) and thus it is not surprising that HRT has parallel effects on these variables. One study reported a decrease in fibrinogen of 0.21 g/l in women on combined transdermal HRT (53) but this was not confirmed by others (54). No data exist on the effect of transdermal HRT on PV. The lower fibrinogen (-0.4 g/l) and PV (−0.039 mPa·s) levels seen in women on combined HRT are of the same order as changes associated with a significantly decreased risk in CHD (55, 56).

The observed effects of estrogens on acute phase protein markers of inflammation could reflect direct actions on their hepatic synthesis. No direct effects of estrogens on hepatocyte synthesis of acute phase proteins *in-vitro* have yet been reported, but plasma values of a range of proteins of hepatic origin are increased by oral estrogen therapy, including HDL (39, 57), sex hormone-binding globulin (58), thyroid

binding protein (58), coagulation factor VII (28, 39) and PAI-1 (28, 39, 59), whilst concentrations of antithrombin III (28) and LDL (39, 57) are decreased. Those studies demonstrated also that transdermal estrogens have no such effect, possibly because they avoid the first pass delivery of oral estrogens from the gut to the liver. Furthermore, as shown here, plasma estrogen levels are considerably lower after transdermal application compared to oral application. The absence of an association of transdermal estrogen therapy with CRP, fibrinogen and PV in the present study is consistent with this concept. However, the plasma concentration of serum amyloid P component (SAP), the protein most closely related to CRP, is lower in women (60, 61), whilst in the Syrian hamster SAP values are exquisitely estrogen sensitive being 100 fold higher in females (62). It is therefore entirely possible that human CRP synthesis may be directly up-regulated by estrogens.

An alternative, or additional, mode of action of estrogens may involve modulation of inflammation itself and/or the cytokine cascade that links it to the acute phase response. Estrogens have been shown to decrease tumor necrosis factor-α, interleukin-1 (63), and increase interleukin-6 (64), and in general to have an anti-inflammatory effect on the endothelium (64, 65). However, hepatic production of fibrinogen and CRP is stimulated by different qualitative cytokine patterns (66) and this may account for the opposite effects on these parameters seen in this study, and in others (67).

The present study has several limitations and thus conclusions have to be drawn very carefully. First, most epidemiological studies in this field are hampered by the fact that the effects of HRT may vary substantially with dose, preparation, route of administration and character of the progesterone component. These variables have often not been adequately characterized. Although, we carefully separated groups according to the progesterone component and route of administration, some of our conclusions are limited by the small numbers in treatment groups, in particular in those on transdermal estrogen therapy. Second, a cross-sectional study does not provide the time-sequence of events and cannot therefore allow conclusions about causality. Effects of HRT on CRP, fibrinogen and PV similar to those reported here have been demonstrated in prospective placebo-controlled studies (24, 29, 39), although the increase in CRP in women on oral estrogen therapy may only be transient (68). Third, our study design is susceptible to confounding in general and particularly to confounding by indication (69). However, adjustment was performed for a variety of potential confounders which were carefully assessed. In contrast, the major strength of this study is that it provides results from a large population-based sample.

In summary, we found higher CRP concentrations and lower fibrinogen and PV values in women on oral estrogen HRT. Transdermal estrogen therapy was not associated with significant differences in either CRP, PV or fibrinogen values compared to no HRT. There is thus no evidence from the present study of a systemic pro-inflammatory effect of HRT. Rather it seems that estrogen therapy alone, in particular when given by mouth, upregulates CRP production. The significance of this effect with regard to cardiovascular risk remains to be established.

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