Effects of hormone replacement therapy on platelet membrane fatty acid composition

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Abstract

Oestrogen replacement therapy has been shown to protect postmenopausal women from ischaemic heart disease, strokes and hypertension. The mechanism of protection conferred by oestrogen, although partly attributable to changes in serum lipoproteins, is not fully understood. The present study was undertaken to assess the effect of hormone replacement therapy on the composition of platelet membrane fatty acids in postmenopausal women. These were analysed by gas-liquid chromatography before and six weeks after continuous conjugated equine oestrogen therapy (0-625 mg daily) combined with cyclical therapy with 75 μg l-norgestrel from day 17 to 28 of a 28-day cycle. Each subject acted as her own control. The principal findings of the study were that, following treatment, there was a 16.2% reduction in platelet membrane polyunsaturated fatty acids (P<0.001), an increase of 9.1 and 7.1% in saturated fatty acids and monounsaturated fatty acids respectively (P<0.001) and a 17.8% reduction in arachidonic acid (P<0.003). There was no correlation between changes in membrane fatty acids and serum lipoproteins. This suggests that the changes in membrane composition noted in this study may be a primary effect of hormone replacement therapy, especially oestrogen.

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Introduction

In postmenopausal women, oestrogen has been shown to exert a cardioprotective effect independent of age, cigarette smoking, or the presence of diabetes mellitus, hypertension, hypercholesterolaemia or obesity (Sullivan et al. 1988). One of the ways in which oestrogen may exert its cardioprotective effect is by influencing the levels and metabolism of fatty acids. For example, the production of prostacyclin, an inhibitor of platelet aggregation and a potent vasodilator, has been shown to be significantly decreased in uterine arteries in postmenopausal women (Steinleitner et al. 1989). Since prostacyclin is a derivative of arachidonic acid, it is possible that oestrogens may affect prostacyclin production via an effect on arachidonic acid metabolism. Arachidonic acid metabolism, especially in platelet membranes, has been shown to be important in coronary artery disease (Wood et al. 1987).

There is little information about the effect of oestrogen on platelet membrane fatty acid composition; this study was undertaken to examine the possibility that changes may be present in postmenopausal women and that these may be reversible with oestrogen treatment.

Materials and Methods

Subjects

Thirty one women were recruited and consented to the study which had been approved by the Ethical Committee at Epsom General Hospital. All subjects had stopped menstruating for at least three months and/or had symptoms of the menopause. All had elevated levels of serum luteinizing hormone (LH; >25 iu/l), follicle-stimulating hormone (FSH; >25 iu/l) and virtually undetectable 17β-oestradiol (<0.1 nmol/l). None of the subjects was on any form of hormone replacement therapy (HRT) at the time of recruitment. No subject had any known major health problems except for one subject with diabetes requiring insulin therapy; five subjects were smokers and twenty-six subjects were non-smokers. Alcohol consumption between 10–14 units, 5–9 units and <5 units per week was noted in 7, 2 and 22 subjects respectively. Subjects were counselled to adhere to alcohol and smoking habits similar to those levels declared prior to initiation of HRT for the duration of the study. A full history was taken and a clinical examination was performed in all subjects prior to enrolment. Each subject was
seen twice, once at the time of recruitment followed by a second visit six weeks later.

At the first visit, blood samples were collected from all subjects after an overnight fast. Twenty millilitres of venous blood was collected into potassium-EDTA glass bottles and subsequently processed for preparation of platelet membranes; plasma was separated for lipoprotein and hormone measurements. All subjects were then commenced on HRT comprising a combination of conjugated equine oestrogens (0·625 mg/day) daily and l–norgestrel (75 μg/day) taken cyclically from days 17 to 28 of a 28-day cycle. The subjects were counselled and encouraged to carry on with their habitual life style and diet for the duration of the study but apart from this no attempt was made to monitor their diet, alcohol intake, smoking and lifestyle in any way.

Assay procedures

Cholesterol was measured by a coupled enzyme reaction (kit from Bio-Stat Reagents, Stockport, Cheshire, UK) on an Encore multiprocessor-controlled centrifugal analyser. High density lipoprotein (HDL)–cholesterol was estimated by measuring cholesterol in the supernatant fraction following polyethylene glycol (6000) precipitation. A coupled enzyme reaction (Bio-Stat Reagent Kit) using an Encore chemistry system was used to measure serum triglycerides. Low density lipoprotein (LDL)–cholesterol was calculated using Friedwald’s formula (Friedwald et al. 1972).

LH and FSH were measured by radioimmunoassay using an 125I label and second antibody phase separation. Oestradiol was extracted from plasma prior to analysis by radioimmunoassay using a tritiated label and charcoal phase separation. The LH and FSH radioimmunoassays were adapted from Hunter & Bennie (1979) and the between–batch coefficients of variation (CV) were 15 and 12±1% at mean concentrations of 30 and 29 iu/l respectively. Cross-reactivity of LH was less than 2% for FSH and thyroid-stimulating hormone and 40% with human chorionic gonadotrophin. The 17β-oestradiol radioimmunoassay is an in-house assay with a between-batch coefficient of variation of 15±3% at a mean concentration of 0·17 nmol/l (D E Fry, personal communication). The cross reactivity profile of the 17β-oestradiol assay is 9±8, 1±2, 0·002, <0·002 and <0·002 for oestrone, oestradiol, testosterone, progesterone and cortisol respectively. The 17β-oestradiol assay is characterised by an ether extraction with a carbonate buffer at a neutral pH of 7 which retains oestrone in the aqueous phase, and uses straight charcoal rather than dextran-coated charcoal for phase separation. All these measurements were based on in-house assays which were performing satisfactorily on external and internal quality control schemes.

Preparation of platelet membranes

Platelets were separated from the rest of the blood components, washed twice with an aqueous solution of cold sodium chloride:sodium EDTA (0·9:1%), lysed with water, washed twice as before, evaporated to dryness under a stream of nitrogen and stored frozen at −50°C.

Analysis of platelet membrane fatty acids

The membrane-bound fatty acids were simultaneously hydrolysed and re-esterified to methyl esters using the anhydrous alkaline methanolysis derivatisation procedure. The free fatty acid methyl esters together with internal standard (C 17:0) were extracted into n-hexane at neutral pH. The extracts were dried under nitrogen, reconstituted in methanol/toluene and analysed by capillary gas-liquid chromatography using a 30 metre DB 225 column as described previously (Barradas et al. 1990). Identification of fatty acids was achieved by direct injection of authenticated fatty acid methyl esters as well as free fatty acid standards. The sum of all fatty acid peaks between the relative retention times defined by C 16:0 and C 22:6 (which included all the fatty acids discussed in this study) was considered to be 100%. Individual fatty acid peaks were quantitated as a percentage of this total. The sensitivity of the analysis was adjusted such that fatty acids with relative proportions greater than 0·12% of the total were included in the quantitation. The fatty acids that achieved quantitation accounted for 95–98% of the total (including C 17:0, an internal standard, the relative concentration of which was <5±0%). The within-batch CV for palmitic, stearic, oleic, linoleic, di-homo-γ-linoleic (DGLA) and arachidonic (AA) acids were 3·7, 1·7, 2·6, 4·6 and 1·2% respectively. The pre- and post-HRT paired samples from each subject were analysed in the same batch adjoining each other in the analytical run.

Measurement of plasma membrane fatty acids requires a particularly careful methodological approach; both inadequate processing and inappropriate storage of plasma membrane preparations have been shown to result in loss of polyunsaturated fatty acids (PUFA) including arachidonic acid (Taylor et al. 1987). Every care was taken by the measures described above to avoid artifactual changes in the present study. Tests for stability of deep frozen pooled platelet preparations performed in the laboratory have given excellent reproducibility for up to 3 years. No unidentified or artifactual peaks appeared on the chromatograms. It is, therefore, unlikely that the changes in PUFA noted in this study were artifactual.

Statistical methods

Statistical analysis was performed using Student’s t-test on the Statworks software of the Apple Mackintosh. Simple linear regression on the same software was performed to
Table 1 Changes in platelet membrane fatty acids in postmenopausal women after HRT

<table>
<thead>
<tr>
<th>Saturated &amp; mono-unsaturated fatty acids</th>
<th>Pre HRT (mean ± s.d.)</th>
<th>Post HRT (mean ± s.d.)</th>
<th>Pre minus post HRT</th>
<th>Change relative to pre HRT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic 16:0</td>
<td>15.7 ± 1.6</td>
<td>17.6 ± 2.1</td>
<td>−1.9**</td>
<td>12.4</td>
</tr>
<tr>
<td>Palmitoleic 16:1</td>
<td>0.38 ± 0.09</td>
<td>0.43 ± 0.12</td>
<td>−0.05*</td>
<td>13.2</td>
</tr>
<tr>
<td>Stearic 18:0</td>
<td>21.7 ± 1.91</td>
<td>23.2 ± 2.48</td>
<td>−1.51**</td>
<td>7.2</td>
</tr>
<tr>
<td>Oleic 18:1 n9</td>
<td>17.9 ± 1.65</td>
<td>18.39 ± 3.98</td>
<td>−0.49</td>
<td>2.7</td>
</tr>
<tr>
<td>Vaccenic 18:1 n7</td>
<td>1.16 ± 0.19</td>
<td>1.28 ± 0.19</td>
<td>−0.12**</td>
<td>10.3</td>
</tr>
<tr>
<td>Arachidic 20:0</td>
<td>1.07 ± 0.12</td>
<td>1.15 ± 0.16</td>
<td>−0.08</td>
<td>7.5</td>
</tr>
<tr>
<td>C 13-eicosatrienoic 20:1 n7</td>
<td>0.78 ± 0.2</td>
<td>0.84 ± 0.21</td>
<td>−0.06</td>
<td>7.7</td>
</tr>
</tbody>
</table>

Polyunsaturated fatty acids

| Linoleic 18:2 n6                         | 6.06 ± 1.11           | 5.57 ± 1.04            | 0.49**             | 8.1                            |
| DGGLA 20:3 n6                            | 1.54 ± 0.36           | 1.32 ± 0.3             | 0.22**             | 14.3                           |
| Arachidonic 20:4 n6                      | 22.7 ± 3.3            | 18.6 ± 4.0             | 4.05**             | 17.8                           |
| Eicosapentaenoic 20:5 n3                 | 0.54 ± 0.18           | 0.41 ± 0.16            | 0.13**             | 24.1                           |
| Docosatetraenoic 22:4 n6                 | 1.72 ± 0.33           | 1.68 ± 0.37            | 0.04               | 2.3                            |
| Docosapentaenoic (DPA) 22:5 n3            | 1.59 ± 0.36           | 1.17 ± 0.35            | 0.42**             | 26.4                           |
| Docosahexaenoic (DHA) 22:6 n3             | 1.55 ± 0.44           | 1.22 ± 0.39            | 0.33**             | 21.3                           |

Sum fatty acids groups

| Sum n9                                  | 17.9 ± 1.7            | 18.4 ± 4.0             | −0.5               | 4.0                            |
| Sum n6                                  | 32.3 ± 4.2            | 27.4 ± 4.9             | 4.9**              | 15.1                           |
| Sum n3                                  | 3.7 ± 0.8             | 2.7 ± 0.9              | 1.0**              | 27.0                           |
| Sum saturates                           | 37.4 ± 3.0            | 40.8 ± 4.3             | −3.4**             | 9.1                            |
| Sum mono-unsaturates                    | 20.2 ± 1.9            | 21.6 ± 2.3             | −1.4**             | 7.1                            |
| Sum polyunsaturates                     | 35.9 ± 4.7            | 30.1 ± 5.6             | 5.8**              | 16.2                           |

Fatty acid ratios

| Stearic/oleic                           | 1.23 ± 0.1            | 1.22 ± 0.12            | 0.01               | 1.1                            |
| Sum n9/sum (n6 + n3)                    | 0.51 ± 0.14           | 0.67 ± 0.2             | −0.16**            | 31.4                           |
| DGGLA/Arachidonic (Δ-5 desaturase)       | 0.069 ± 0.02          | 0.073 ± 0.02           | −0.004             | 5.8                            |
| DPA (22:5):DHA (22:6) (Δ-4 desaturase)   | 1.13 ± 0.29           | 1.53 ± 0.44            | −0.4**             | 35.8                           |

*P<0.05; **P<0.001: significant differences between pre and post HRT values. DGGLA, di-homo-γ-linoleic acid.

study the relationship between groups of data. Differences between results, assessed by paired Student's t-test, were considered significant if the value of P was less than 0.05.

Results

Subjects

The mean age of subjects in the study (± s.d.) was 51 ± 6 years (range 32 to 63 years). The mean (± s.d.) duration of amenorrhea prior to the study was 3.2 (± 3.5) years.

Platelet membrane fatty acids (Table 1)

Saturated fatty acids (SFA) The mean proportions of palmitic and stearic acids increased significantly after 6 weeks of HRT (P<0.002).

Mono-unsaturated fatty acids (MUFA) The proportions of palmitoleic and vaccenic acids increased significantly after HRT (P<0.01 and 0.002 respectively). The increase in platelet membrane oleic acid did not achieve statistical significance.

Polyunsaturated fatty acids (PUFA) As a proportion of total membrane lipids, the relative levels of all PUFA in platelet membranes decreased significantly after 6 weeks of HRT. This included both n3 PUFA and n6 PUFA (P<0.003 for all). For arachidonic acid, this represented a decrease from 22:7 to 18.6% of total lipids after HRT, a relative change of 17.8%, and for linoleic acid a decrease from 6.1 to 5.6%, a relative change of 8.1% (Fig. 1a).

The total saturated fatty acid content of membranes increased from 37.4% to 40.8% following HRT, while total MUFA content increased from 20.2% to 21.6% (P<0.001 for each).

The sum of n6 PUFA and of n3 PUFA both showed a marked fall after six weeks of HRT from 32.3 to 27.4% and from 3.7 to 2.7% respectively (P<0.001 for both).

The ratio of docosapentaenoic acid:docosahexaenoic acid (DPA:DHA) can be used as an indication of the
activity of the enzyme δ-4 desaturase. The increase in the
DPA:DHA ratio from a mean of 1:13 prior to HRT to
1:53 six weeks after HRT (P<0.001) suggests a decrease
in the activity of the enzyme.

**Serum lipoproteins**

The mean concentrations of serum total cholesterol and
calculated LDL-cholesterol fell from 7.1 and 4.64 to 6.43
and 3.65 mmol/l respectively after therapy (P<0.001)
(Fig. 1b). All subjects had triglyceride levels below
5.5 mmol/l before treatment. Mean triglyceride levels
increased significantly from 1.19 to 1.48 mmol/l after
HRT (P<0.05). There was no significant change in the
level of HDL-cholesterol.

There was no correlation between any of the changes in
serum lipoproteins and membrane fatty acids (Table 2).

**Discussion**

The proportions of palmitic, stearic, oleic, linoleic and
arachidonic fatty acids in platelet membranes were similar
to those described by previous workers (Wood et al. 1987,
Kinsella 1990). The main findings of this study indicate a
change in platelet membrane fatty acid composition in
response to HRT treatment, with an overall increase in
the proportion of saturated and mono-unsaturated fatty
acids (+9.1 and +7.1% respectively) and a decrease in the
proportion of n3, n6 and total polyunsaturated fatty acids
(-27%, -15% and -16.2% respectively). From a physiological
point of view, we believe that the most important
platelet membrane fatty acid change was the observed
reduction in arachidonic acid and the possible significance
of this change is discussed later. The change in the relative

![Figure 1](https://via.placeholder.com/150)

**Figure 1** Changes in (a) membrane polyunsaturated fatty acids
(linoleic acid, 18:2, n6; di-homo-gamma-linoleic acid (DGLA), 20:3, n6;
arachidonic acid (AA), 20:4, n6) and (b) serum lipids in
postmenopausal women before (open bars) and after (solid bars)
hormone replacement therapy (HRT). *P<0.05, **P<0.001
significantly different compared with pre HRT.

<table>
<thead>
<tr>
<th>Table 2 Correlation between changes in serum lipoprotein lipids and changes in platelet membrane fatty acids</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Platelet membrane fatty acids</strong></td>
</tr>
<tr>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>vs total cholesterol</td>
</tr>
<tr>
<td>Sum polyunsaturated fatty acids</td>
</tr>
<tr>
<td>vs total cholesterol</td>
</tr>
<tr>
<td>vs triglycerides</td>
</tr>
<tr>
<td>Sum mono-unsaturated fatty acids</td>
</tr>
<tr>
<td>vs triglycerides</td>
</tr>
</tbody>
</table>

r, correlation coefficient; calc, calculated.
proportions of the fatty acids suggests decreases in the activities of both \( \delta-4 \)- and \( \delta-5 \)-desaturase activities.

It is unlikely that the changes observed were due to day-to-day fluctuations in diet and life-style; subjects were encouraged to maintain the same life-style (including diet, smoking and alcohol consumption) between the platelet membrane measurements. In addition, previous studies have shown very little day-to-day fluctuation in platelet membrane fatty acid composition (Mori et al. 1987).

The changes in PUFA of the n3 and n6 series (with the exception of linoleic acid) could be due to (i) decreased desaturation and/or chain elongation (Kinsella 1990), (ii) increased formation of biologically important products such as eicosanoids, (iii) increased catabolism by the \( \beta \)-oxidative pathway (Okuyama & Sakai 1991) or decreased incorporation of essential PUFA such as linoleic acid (LA) and \( \alpha \)-linolenic acid. A reduction in PUFA due to conversion to biologically active products cannot be excluded since we did not measure products of PUFA metabolism. Neither can preferential catabolism of PUFA through \( \beta \)-oxidation, especially of n3 PUFA, be discounted as the cause of the low PUFA observed.

The reduction in the membrane content of linoleic acid, an essential fatty acid consumed in the diet, would suggest either a decreased dietary intake or reduced incorporation into the membrane (Okuyama & Sakai 1991). As discussed above, a reduction in dietary intake is considered unlikely. Reduced LA in the membrane could also account, at least in part, for reduction in the levels of other n6 PUFA.

A further possible mechanism for the observed changes in membrane fatty acids is that they may be secondary to changes in the cholesterol content of the membrane reflecting a change in circulating levels of total or LDL-cholesterol (induced by oestrogen therapy) or a direct membrane effect of oestrogens. The changes in serum lipoprotein lipids shown by us are similar to those described by others. Miller et al. (1991) used conjugated oestrogens at a similar dose to ours along with 150 \( \mu \)g \( \Delta_1 \)-norgestrel (equivalent to 75 \( \mu \)g \( \Delta_1 \)-norgestrel) and although they measured serum lipoprotein lipids at 3 months rather than at 6 weeks as in the present study, they showed a non-statistical increase in triglycerides and HDL-cholesterol and a significant fall in serum total and LDL-cholesterol. However, Fletcher et al. (1991) who used the combination of conjugated oestrogens with cyclical norgestrel at identical doses to those in our study showed almost identical serum lipoprotein lipid results to those described by us in the present study at 6 weeks. Decreases in total and LDL-cholesterol have been shown to be associated with a relative increase in SFA and decrease in PUFA in patients with renal failure on haemodialysis, and after plasmapheresis in subjects with familial hypercholesterolaemia (Brook et al. 1983, Peuchant et al. 1990). Although this mechanism remains a possibility, no correlation between the changes in platelet membrane fatty acids and those in serum lipoproteins was observed in this study.

Alternatively, the changes in membrane fatty acids may be due to a direct effect of oestrogen causing a decrease in PUFA as the primary event resulting from either decreased incorporation of essential PUFA and/or inhibition of fatty acid desaturase(s) and/or elongase. As assessed by the DPA:DHA and DGLA:AA ratios, there was an apparent decrease in the activity of \( \delta-4 \)-desaturase (and to a lesser extent \( \delta-5 \)-desaturase) following HRT suggesting the possibility of a direct effect of oestrogen on the desaturase/elongase enzyme systems.

More than 90% of \( \Delta_1 \)-norgestrel has been shown to be excreted virtually completely within 4 days following drug administration (Sisenwine et al. 1975). The half life of \( \Delta_1 \)-norgestrel has been shown to be approximately 24 h and less than 1% is found in the body after 5 to 6 days. Furthermore, storage of \( \Delta_1 \)-norgestrel in body tissues is negligible and unimportant (Fotherby 1970). We therefore believe that platelet membrane composition assessed in this study coincides with the period of oestrogen dominance. Since the life span of platelets in health is approximately seven days (Oates et al. 1988), the changes seen in this study may predominantly reflect the the effect of the oestrogen component of the HRT. Platelet membranes contain a high proportion of arachidonic acid which is largely converted into thromboxane A2. Since most human diets contain only small amounts of arachidonic acid, the majority of this fatty acid in the platelet must be derived indirectly from dietary linoleic acid. The desaturation and elongation of linoleic acid is carefully regulated so that an increase in dietary consumption beyond a threshold does not lead to any further increase in arachidonic acid (Sanders 1983). It has been postulated that an increase in platelet arachidonic acid may predispose to a number of disease processes by enhancing the generation of eicosanoids and other mediators of tissue injury (Kinsella 1990). Thus, the observed decrease in platelet arachidonic acid content in response to HRT may be a beneficial change.

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