Non-protein bound oestradiol, sex hormone binding globulin, breast cancer and breast cancer risk
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Summary It has recently been found by various authors that despite a normal serum concentration of oestradiol (E2), the percentage of non-protein-bound or free E2 is abnormally high in breast cancer patients. Since it is the free E2 which is considered to be biologically active, confirmation of this finding would be most relevant to the pathogenesis of breast cancer. Using Hammonds's centrifugal ultrafiltration dialysis method we have measured free E2 in heparinized plasma from 68 premenopausal women (a) at high familial risk of breast cancer (n=18), (b) with benign breast disease (n=17), (c) cured of T1N0M0 breast cancer at least 6 months previously (n=17) and (d) normal controls matched for age, parity and Quetelet index (n=16). Sex hormone binding globulin (SHBG) was measured as [3H]-dihydrotestosterone binding capacity. Free E2 and SHBG were also measured in the serum of (e) postmenopausal patients having breast cancer (n=38) and (f) matched control cancer patients (n=67). We confirmed a very good inverse correlation between log free E2 per cent and log SHBG (P<0.0001). The regression lines for groups (a)-(d) were not statistically different. The regression lines for groups (e) and (f) were identical and ran nearly parallel to those for groups (a)-(d) though somewhat lower. This small difference may be ascribed to menopausal status.

Therefore, we found no difference in free E2 percentage, calculated free E2 concentration or SHBG between premenopausal women at risk, women with benign breast disease, patients cured for early breast cancer or having breast cancer and matched controls. However, postmenopausal breast cancer patients had a significantly higher total serum E2 concentration and, by consequence a higher calculated free E2 concentration compared to the carefully matched control group.

Endocrine factors are thought to play a role in the pathogenesis of cancer of the breast and various other hormone responsive organs in man. As blood levels of hormones are considered to be representative of their direct influence on target cells, more sensitive and direct immunological assay methods applied to serum or plasma have largely replaced the determination of urinary hormone metabolites. Oestrogens, progestins, weak androgens and lactogenic hormones like prolactin have been the main topic of the endocrine investigations in breast cancer. The results of all the research performed to date are conflicting. No unequivocal hormonal abnormality could be discovered in patients with breast cancer or in women at risk. It was therefore most exciting when Siiteri et al. (1981), published data on the elevated non-protein bound oestradiol (free E2) fraction in the serum of breast cancer patients. This finding appeared to be most relevant to the alleged promoter function of E2, despite the existence of normal serum concentrations of total E2, since it is the free E2 fraction which is supposed to be biologically active.

We have measured both the free E2 fraction as a percentage of the total E2 concentration, and the actual E2 concentration in the blood from women at risk of breast cancer, breast cancer patients and matched controls. Our findings do not support the hypothesis that the pathogenesis of breast cancer is related to an elevated free fraction of the total E2 concentration in blood. However, the free E2 concentration, as calculated from the total E2 concentration and the free E2 fraction is shown to be abnormally high in postmenopausal breast cancer patients as a consequence of their elevated total serum E2 concentration.

Subjects and methods

Subjects

Serum samples were obtained from 38 postmenopausal breast cancer patients and a control group of 67 women admitted for malignant lymphoma (n=21), melanoma (n=5), cancer of the lung (n=21) or large bowel (n=20). The two groups were matched for age, parity and Quetelet index (in Kg m⁻²) as shown in Table I. All women were clinically judged to have normal thyroid function.

Heparinized plasma was collected by continuous venous sampling from 68 premenopausal women belonging to one of the following 4 groups: 18 women at a high familial risk for breast cancer, i.e. mother and at least one sister having breast cancer (group R), 17 women curatively treated for early (T1N0M0) breast cancer at least 6 months ago (group C), 17 women with histologically defined
benign proliferative breast disease (group B) and 16 normal women (group N), matched for age, parity and Quetelet index, as shown in Table II. These premenopausal women were known to have normal thyroid function parameters and to use no contraceptive pill or any other drug.

Serum and heparinized plasma were collected in Venoject® glass tubes simultaneously from 24 healthy adult female and male individuals for comparison of assay results in serum and plasma.

### Table II Matching parameters for 68 premenopausal women (mean values ± s.d.)

| Group | R (n=18) | C (n=17) | B (n=17) | N (n=16) |
|-------|----------|----------|----------|----------|
| Age (yr) | 38.7±7.6 | 40.1±3.7 | 36.4±5.0 | 38.1±7.4 |
| Parity | 2.1±2.1 | 1.9±1.7 | 1.2±1.1 | 1.8±1.3 |
| Quetelet index (kg m⁻²) | 2.4±0.3 | 2.1±0.3 | 2.2±0.2 | 2.3±0.2 |

### Methods of blood sampling

Single serum samples were drawn on first admission in Venoject glass tubes from the 38 patients with breast cancer and the 67 cancer patients serving as a matched control group.

Heparinized plasma was collected over at least 7 h by a continuous venous sampling technique in the premenopausal groups R, C, B and N. This technique gave the opportunity to observe diurnal variation of plasma concentrations in 20 min interval samples. For the determination of the total E₂ concentration and the percentage of free E₂, 21 subsequent 20 min plasma samples from each individual women were pooled. All women were investigated between Days 18 and 24 of their menstrual cycle. The plasma progesterone concentration had to be >10 nmol l⁻¹ as evidence of an active corpus luteum. The procedure is part of a larger investigation of hormonal patterns and breast cancer risk (Bruning et al., 1984).

All samples had been stored at -20°C (maximum 3 years) and had not been thawed before use.

### Analytical methods

Total E₂ concentration was measured by a conventional radioimmunoassay. Serum extraction with ether was followed by Sephadex LH-20 chromatographic separation. A highly specific antiserum raised against E₂-6-0-carboxy-methyl-oxime-bovine serum albumin was used (Bulbrook et al., 1978). Progesterone was measured with a commercial radioimmunoassay-kit (Farmos, Turku, Finland).

The percentage of free E₂ was measured by centrifugal ultrafiltration dialysis of undiluted serum or plasma at +37°C (Hammond et al., 1980). The interassay and intraassay coefficients of variation were 12 and 8.6% respectively.

Sex hormone binding globulin (SHBG) was measured as [³H]-dihydrotestosterone ([³H]-DHT) binding capacity. In the assay method concanavalin A-sepharose was used to separate unbound [³H]-DHT and [³H]-DHT bound to SHBG; transcortin binding capacity was saturated with cortisol (modified after Nisula and Dunn, 1979). Albumin was measured colorimetrically using bromocresyl purple (Pinnel & Northam, 1978).

Body fat mass was determined in 18 healthy individuals by measuring body weight and the thickness of 4 standard skinfolds with a Harpenden skinfold caliper (Durnin & Womersley, 1974).

### Results

As can be seen from Table III we have found no significant differences for SHBG or free E₂ percentage when comparing the assay results of the serum samples from the postmenopausal breast cancer patients and their matched controls. However, the mean total E₂ concentration in the postmenopausal breast cancer patient group was significantly higher (P=0.02) after logarithmic transformation had been applied to correct for the skewed distribution of total E₂ values.

Similarly, the calculated free E₂ concentration was significantly higher in the postmenopausal breast cancer patients than in the control cancer patients (P=0.01). No such difference could be demonstrated between the premenopausal groups. The mean albumin concentration in the postmenopausal breast cancer patients was slightly elevated (P<0.01) compared to that in the matched control cancer patients.

The data in Table IV indicate that no significant differences between groups were found in the heparinized plasma samples obtained from the premenopausal women of groups R, B, C and N.

A very good correlation between SHBG and free E₂ percentage was observed (P<0.0001). Analysis of
Table III  Serum values (mean ± s.d. resp. mean of log-transformed values) in postmenopausal women with breast cancer (n=38) and female control patients (n=67).  

|                      | Breast cancer group | Control group | P-value<sup>a</sup> |
|----------------------|---------------------|---------------|---------------------|
| Total $E_2$ (pmol$^{-1}$) | 155.5 ± 314.3       | 73.4 ± 127.3  | NS                  |
| Log (total $E_2$)     | 1.77                | 1.52          | $P=0.02$            |
| Free $E_2$ (%)        | 1.54 ± 0.38         | 1.47 ± 0.37   | NS                  |
| Log (free $E_2$%)     | 0.17                | 0.15          | NS                  |
| Calculated free $E_2$ (pmol$^{-1}$) | 2.08 ± 4.09 | 1.11 ± 2.08 | NS                  |
| Log (calculated free $E_2$ pmol$^{-1}$) | 1.95               | 1.68          | $P=0.01$            |
| SHBG (nmol DHT$^{-1}$) | 44.5 ± 28.1         | 44.8 ± 18.9   | NS                  |
| Log (SHBG nmol DHT$^{-1}$) | 1.58               | 1.61          | NS                  |
| Albumin (g$^{-1}$)    | 41.49 ± 5.02        | 39.93 ± 4.92  | $P<0.01$            |

<sup>a</sup>Paired t-test.

Table IV  Plasma values (mean ± s.d.) in premenopausal women.<sup>a</sup>  

| Group               | $R$ (n=18) | $C$ (n=17) | $B$ (n=17) | $N$ (n=16) |
|---------------------|------------|------------|------------|------------|
| Total $E_2$ (pmol$^{-1}$) | 366.8 ± 126.5 | 349.9 ± 162.4 | 287.1 ± 77.0 | 331.1 ± 127.3 |
| Free $E_2$ (%)       | 1.78 ± 0.28 | 1.86 ± 0.39 | 1.84 ± 0.34 | 1.70 ± 0.54 |
| Calculated free $E_2$ (pmol$^{-1}$) | 6.3 ± 1.6    | 4.9 ± 1.4   | 5.0 ± 1.5   | 5.0 ± 1.6   |
| SHBG (nmol$^{-1}$)   | 35.9 ± 11.3  | 33.9 ± 12.1  | 31.6 ± 10.3  | 37.1 ± 20.6 |
| Albumin (g$^{-1}$)   | 34.5 ± 3.58  | 35.12 ± 2.26 | 34.95 ± 2.21 | 33.89 ± 1.79 |

<sup>a</sup>Due to 0.8× dilution with heparin-saline solution values are lower than may be expected in serum.

Discussion

Moore et al. (1982) gave support to Siiteri’s preliminary data showing that in premenopausal women with stage 2 breast cancer serum $E_2$ concentrations were normal, but free $E_2$ concentrations (not only percentages of the total $E_2$ concentrations) were significantly elevated. In postmenopausal patients these investigators found both total and free $E_2$ to be abnormally high. Reed et al. (1983) confirmed that the free $E_2$ fractions of the total $E_2$ concentration in the plasma of postmenopausal women with breast disease were elevated.

We cannot support these findings as our results do not demonstrate any elevation of the free $E_2$ fraction in women with premenopausal or postmenopausal breast cancer or premenopausal benign breast disease compared to control values. Premenopausal women with a relatively high risk of breast cancer because of their family history or of previous breast cancer showed similarly normal free $E_2$ fraction values. The total $E_2$ concentration was found to be significantly elevated in the postmenopausal breast cancer patient group compared to the control group which was composed of patients with other cancers, carefully matched for age, parity and Quetelet’s body mass index. This
finding is consistent with data obtained by Moore et al. (1982).

Although Reed et al. (1983) used an equilibrium dialysis technique, Moore et al. (1982) and Siiteri et al. (1981) used the same centrifugal ultrafiltration dialysis method as we did to determine the free E_2 fraction (Hammond et al. (1980). There were no differences in the [³H]-DHT binding capacity of SHBG or albumin concentrations between our groups which could explain the discrepancies between our data and the free E_2 results obtained by Moore et al. and Reed et al. The difference of albumin concentrations between our postmenopausal patient groups would rather be in favour of a smaller free E_2 fraction in breast cancer patients, as serum albumin represents a major compartment of protein-bound E_2.

We could confirm the strong inverse correlation between SHBG and free E_2 percentage (not free E_2 concentration) as was demonstrated before (Siiteri et al., 1981, Moore et al., 1982, Reed et al., 1983). However, we have no indication that, as Moore et al. (1982) concluded, for a given SHBG concentration less E_2 would be bound in breast cancer patients than in the control population since the regression lines for log free E_2 percentage vs log SHBG are identical for our breast cancer groups and their respective matched control groups.

A small, but statistically significant difference in free E_2 percentage was observed between the premenopausal plasma samples and the postmenopausal serum samples. This difference could not be ascribed to the difference between serum and heparinized plasma itself as shown in Figure 3.

An inverse correlation between SHBG and excess
body weight was reported by De Moor & Joosens (1970) and more recently confirmed in massively obese women (Kopelman et al., 1980) and in postmenopausal women with endometrial cancer (Nisker et al., 1980). However, we could not find significant correlations between free E₂ percentage and age, body weight or body fat mass respectively. We conclude that some other factor(s), possibly related to the menopausal status, may be involved.

If free E₂ in blood is to be regarded as important since it represents the biologically active fraction of the total E₂ concentration, then the free E₂ concentration, not the mere free E₂ fraction expressed as a percentage of the total E₂ concentration should be considered. Our data do not support a role for a

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