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Salivary Oestradiol and Progesterone Levels in Premenopausal Women with Breast Cancer

D.Y. WANG, V.E. FANTL, F. HABIBOLLAHI,* G.M.G. CLARK, I.S. FENTIMAN,* J.L. HAYWARD* and R.D. BULBROOK

*Department of Clinical Endocrinology, Imperial Cancer Research Fund, Lincoln's Inn Fields, London, WC2A 3PX, U.K. and *I.C.R.F. Breast Cancer Unit, Guy's Hospital, London, SE1 9RT, U.K.*

Abstract—*The concentrations of oestradiol and progesterone have been measured in salivary specimens collected daily over a complete menstrual cycle in* 12 *patients with operable breast cancer and* 12 *normal control volunteers. There was no significant difference* ($P > 0.05$) *for either hormone between these two groups. Both showed a mid-cycle rise in oestradiol levels followed by a smaller but sustained increase during the luteal phase. The progesterone concentration increased markedly during the luteal phase of the cycle.*

Total or non-protein bound oestradiol levels measured in blood samples from 19 *normal women were both linearly correlated* ($P < 0.001$) *with the concentration of oestradiol in matched saliva samples. The amount of free oestradiol in blood was about twice that found in saliva.*

INTRODUCTION

THERE IS a great deal of circumstantial evidence indicating that oestrogens are of importance in the aetiology of breast cancer [1]. Nevertheless, measurement of these hormones in blood and urine has not been of value in elucidating their role in the disease. However it has been assumed that the total amount of oestrogens in blood, or their metabolites in urine, is directly related to oestrogenic action. The possibility that the binding of oestrogens to proteins in the blood will alter their biological activity has been largely ignored.

More recent studies have examined the role of non-protein-bound, or free, oestradiol in the aetiology of the disease. These investigations have shown that pre and post menopausal patients with breast cancer have significantly higher levels of free oestradiol than controls [2–5] and that women who develop breast cancer have raised free oestradiol before clinical onset [6]. Also, normal Japanese women (at low risk of breast cancer) have almost twice as much oestradiol bound to sex hormone binding globulin as normal British women who are at higher risk [7]. Although the median value for non-protein-bound oestradiol was similar for both races the proportion of oestradiol that was weakly bound to albumin in British women was twice that found for Japanese females.

It has been claimed that one of the advantages of measuring steroid hormones in saliva is the direct equivalence between the amount of biologically available steroid in blood and the concentration of that steroid in saliva [8–10]. If this is so, then the measurement of oestradiol in saliva could be of importance not only in helping to investigate the biology of the disease but also in the identification of women at increased risk.

It has also been claimed that reduced progesterone secretion during the luteal phase of the menstrual cycle leads to an increased risk of developing breast cancer [11] although it has recently been reported that the concentration of salivary progesterone in patients with breast cancer is similar to that found in unaffected women [12]. This present study would, therefore, afford an opportunity to confirm the latter finding.

We have therefore measured the concentrations of oestradiol and progesterone in saliva samples collected daily over a complete menstrual cycle from women with proven breast cancer and from controls, matched for age and parity. In addition the level of salivary oestradiol has been compared with the amount of non-protein-bound, or free, oestradiol in the blood of a group of normal volunteers.

METHODS AND MATERIALS

Subjects

The patients with breast cancer were 12 women who had undergone treatment at least 6 months prior to saliva collections and had no overt evidence of recurrence or metastases. Twelve healthy

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women were used as controls. Both groups of women were experiencing regular menstrual cycles and none had taken oral contraceptives for at least 12 months. Each of these subjects provided daily saliva samples for one complete menstrual cycle according to a written protocol. Volunteers were instructed to rinse out their mouths with tap water, but not to brush their teeth, before saliva collection. At least 5 min was then allowed to elapse before saliva collection commenced to avoid dilution of saliva with water. Saliva samples were tested for the presence of haemoglobin with Labstix (Ames) and none showed blood contamination in excess of 0.01%. The first collection coincided with the first day of the cycle and the date of the next menstrual cycle was recorded. The time of saliva collection was not controlled and was between 9 a.m. and 9 p.m. Details of these two groups are given in Table 1.

In addition to these volunteers, matched blood and saliva samples were taken from a further 19 normal women aged between 20 and 45 years.

Table 1. *Characteristics of patients with breast cancer and unaffected controls*

	Patients	Controls
Age	43.2 ± 2.9*	43.4 ± 2.7
Weight (kg)	62.0 ± 10.5	59.7 ± 5.6
Height (cm)	164.3 ± 5.6	164.2 ± 5.9
Parity	2.42 ± 1.16	2.25 ± 0.91
(Range)	(1–4)	(1–5)
Cycle length (days)	25.3 ± 1.7	25.8 ± 1.6
(Range)	(23–29)	(23–29)
Number	12	12

* Mean ± S.D.

Saliva and blood

Volunteers were given plastic vials into which they collected saliva by direct salivation. Since the specimens were collected at home they were stored in the volunteers' domestic deep-freeze or freezer compartment of the refrigerator. The samples were sent to the laboratory by mail; the posting day being either Monday or Tuesday to avoid delay in the post over weekends. Trial experiments showed that the titre of oestradiol was unchanged in saliva kept at room temperature for up to 3 days. Saliva samples on arrival at the laboratory were stored at −20°C until analysed.

The matched saliva and blood specimens were collected within 15 min of each other. The blood was allowed to clot at room temperature and the serum separated. Saliva and serum samples were stored at −20°C.

Assay of oestradiol in saliva

Saliva (1 ml) was extracted with 3.5 ml of a mixture of analytical grade isopentane and freshly distilled ether (v/v 4 : 3). After freezing the saliva in a bath of solid CO_2/ethanol, the organic phase was decanted into 50 × 13 mm bacteriological glass tubes. The organic solvent was evaporated by placing the tubes in a heating block the temperature being gently raised from 30 to 40°C. The mean recovery of tritiated oestradiol from six saliva samples by this organic solvent was 97% (± 1% S.D.). The amount of oestradiol in extracts was estimated by radioimmunoassay using the monoclonal antibody 6E1 [13] and 3000 cpm of tritiated oestradiol ([2,4,6,7,16,17−^{3}H] oestradiol, 152 Ci/mmol Amersham International plc). The final assay volume was 500 µl and the buffer was physiological saline (pH 7.4) containing 0.1% (w/v) gelatine and 0.1% (w/v) sodium azide. The samples were allowed to equilibrate overnight at 4°C and the unbound radioactive ligand was separated by the addition of 50 µl of an aqueous mixture of dextran (0.08 mg/ml) and charcoal (8 mg/ml) to each assay tube. After mixing, the tubes were kept on ice for 20 min, then centrifuged and tritium counts determined in the supernatant in a Packard PLD-Tricarb PRIAS liquid-scintillation counter. Assay samples for counting were placed into plastic inserts (Sterilin, Cat. No. 505) and 3.5 ml phosphor (Packard Pico-Fluor 15) added. The efficiency was 45% and samples counted until at least 10,000 cpm were accumulated, giving a counting error of 1%.

The sensitivity of the assay was such that a 50% reduction in the binding of radioactive ligand corresponded to 6 pg (22 fmole) on the standard curve. The minimum amount that can be detected with a 95% certainty is 0.7 pg/tube which represents 2.6 pmol/l in the assay.

Accuracy was assessed by measuring oestradiol levels in saliva samples to which known amounts of oestradiol had been added. The results are shown in Table 2.

The specificity of the assay was considered to be satisfactory for the following reasons. The monoclonal antibody 6E1 used in this assay is highly specific having cross-reactions with oestrone and oestriol of only 0.04 and 1.0%, respectively [13]. Furthermore, the mixture of isopentane–ether reduces the amount of polar material extracted. The effectiveness of this can be judged by the fact that 45% of oestriol was recovered in this solvent system compared with over 80% using ether alone.

Table 2. *Accuracy of oestradiol assay*

pg Added	pg Measured	Difference	*n*
0	0.7 ± 0.2*	–	6
2.6	3.0 ± 0.1	2.3	6
5.2	5.6 ± 0.2	4.9	6

* Mean ± S.D.

Finally, the displacement of tritiated oestradiol by varying amounts of saliva extracts was essentially parallel to that of standard oestradiol.

The assays for progesterone and oestradiol were so designed as to attempt to minimise batch bias whilst at the same time keeping to a minimum the number of assays performed. The saliva samples from a complete menstrual cycle from a cancer patient and a non-affected woman were processed together. The determination of salivary steroid in cancer and controls were alternated in such a way that the samples for day 1 of the menstrual cycle of the cancer and control was followed by the saliva samples for day 2 of cancer and control, and so on, until the end of the cycle. In addition the order of cancer and control was alternated such that if the cancers were first in one assay (i.e. odd numbers) then the cancers would be placed second (i.e. even numbers) in the following assay. There were standard curves (in duplicate) at the beginning and half-way through each run. In addition four batches of quality controls were placed evenly throughout each assay.

For the purpose of separation of bound from free ligand the assay was divided into two equal halves and each half processed separately. This minimised the variation due to different times for which charcoal was present.

The inter and intra assay variations based on the two quality control pools for the entire experiment was 15% and 12% (mean 7 pmol/l) and 14% and 10% (mean 10 pmol/l) respectively. Figure 1 shows the accumulated standard curves for these assays.

Assay of progesterone in saliva

Saliva (0.25 ml) was extracted with 4 ml of isopentane in glass-stoppered tubes (Quick-fit MF24/0/4). The preparation of the extract for radioimmunassay was the same as that described for the assay of salivary oestradiol. The mean recovery was 95% (± 3% S.D.) for eight samples.

The radioimmunassay system used the monoclonal antibody 11P27 [14] and 4000 cpm of [1,2,6,7-^{3}H] progesterone (92 Ci/mmol; Amersham International plc). The remainder of the assay was the same as that for oestradiol except that the tubes were incubated at 37°C for 45 min before leaving overnight at 4°C.

Validation of this method has already been described [14].

The inter and intra assay variation based on the two quality control pools for all the assays was 12 and 10% (mean 133 pmol/l) and 14 and 4% (mean 318 pmol/l), respectively. Figure 2 shows the accumulated standard curves for these assays.

Serum oestradiol assay

The total serum oestradiol was determined using a radioimmunoassay method already described [16] except that a specific monoclonal antibody to oestradiol 6E1 [13] was used instead of a rabbit polyclonal antibody.

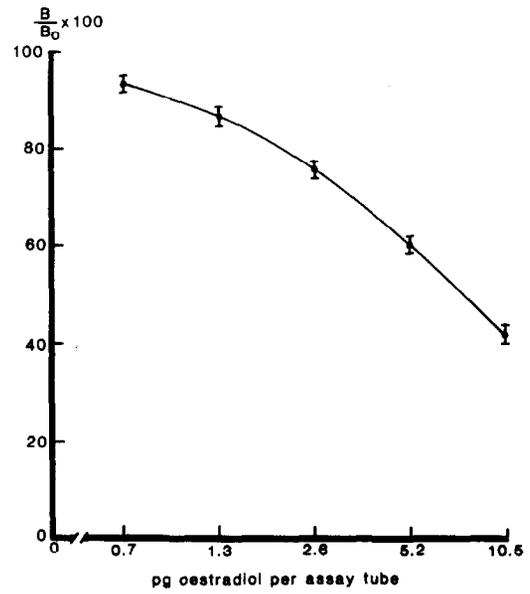

Figure 1. Accumulated standard curves for oestradiol assay. B represents the binding at the given concentration of standard less that for the non-specific binding and B_0 represents the binding at nil concentration of standard less that for the non-specific binding. The scatter bars indicate the S.D. of the 24 standard curves (12 assays) used for measuring salivary oestradiol. 10.5 pg of oestradiol per assay tube represent 38.6 pmol of oestradiol per litre of saliva.

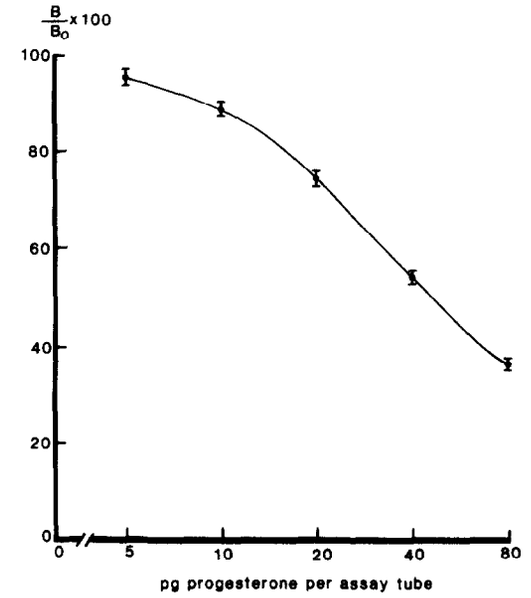

Figure 2. Accumulated standard curves for progesterone assay. B represents the binding at the given concentration of standard less that for the non-specific binding and B_0 represents the binding at nil concentration of standard less that for the non-specific binding. The scatter bars indicate the standard deviation of the 24 standard curves (12 assays) used for measuring salivary progesterone. Eighty picograms of progesterone per assay tube represents 1017 pmol of progesterone per litre of saliva.

Determination of percentage non-protein-bound oestradiol

The proportion of oestradiol bound to blood proteins was determined using a centrifugal ultrafiltration dialysis of undiluted serum at 37°C as described by Hammond *et al.* [17].

RESULTS

Salivary oestradiol and progesterone levels in patients with breast cancer and controls

The mean amounts of salivary oestradiol (Fig. 3) and salivary progesterone (Fig. 4) throughout the menstrual cycle in patients with breast cancer and controls are similar. Both groups of women exhibit the same peak values at day 11 for oestradiol and on days 18 to 22 for progesterone.

The mean amounts of oestradiol for the combined study groups are about 6 pmol/l during the early follicular phase of the cycle (Fig. 5) which rise to a peak value of 10 pmol/l at day 11. After this peak value the mean levels decline for 2 days and then remain at 7–8 pmol/l for most of the luteal phase.

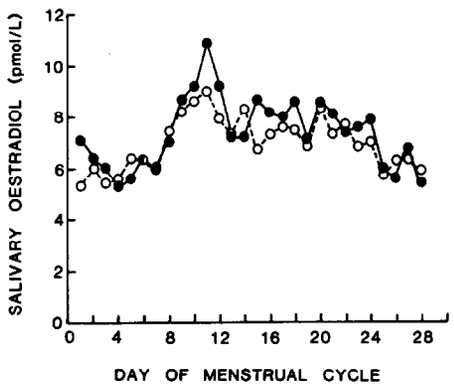

Figure 3. Salivary oestradiol levels during the menstrual cycle for patients with breast cancer and unaffected control women. The daily mean values of salivary oestradiol for 12 patients with breast cancer (o---o) are compared with 12 unaffected women (●---●) matched for age and parity.

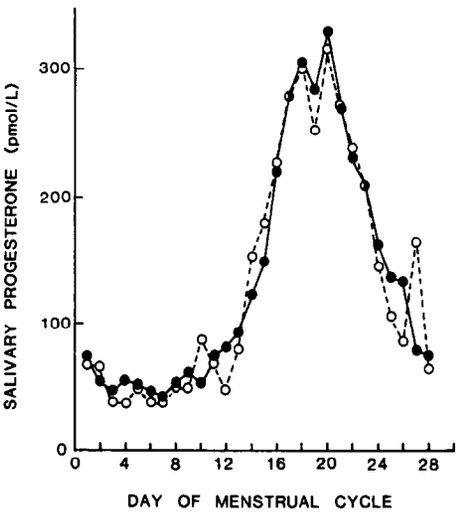

Figure 4. Salivary progesterone levels during the menstrual cycle for patients with breast cancer and unaffected control women. The daily mean values of salivary progesterone for 12 patients with breast cancer (o---o) are compared with 12 unaffected women (●---●) matched for age and parity.

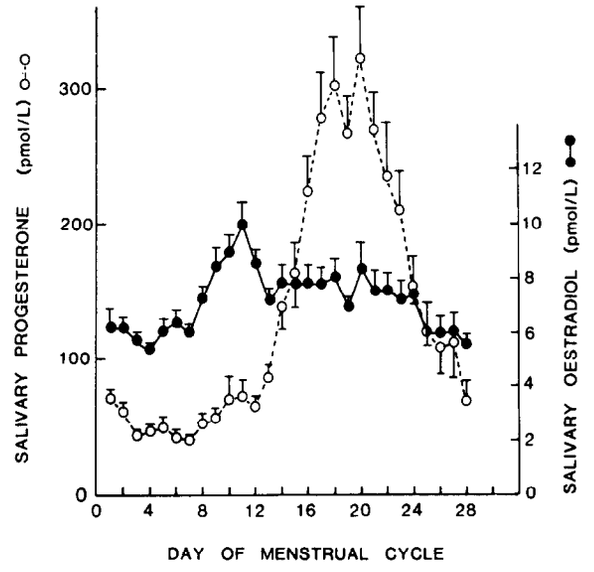

Figure 5. Salivary oestradiol and progesterone levels during the menstrual cycle for the combined breast cancer cases and controls. The levels of salivary oestradiol (●---●) and progesterone (o---o) are the mean values for the combined cases and controls (n = 24). The scatter bars represent the standard errors of the means.

Progesterone levels are about 50 pmol/l during the first half of the menstrual cycle and rise sharply during the luteal phase to reach a peak value in excess of 300 pmol/l at day 20 (Fig. 5). The average amount of progesterone then falls rapidly until menstruation.

Relationship between serum and salivary oestradiol levels

There is a highly significant linear correlation ($r = 0.79$; $P < 0.001$) between salivary oestradiol level and the total amount of the hormone in blood for the 19 volunteers studied (Fig. 6). There was a similarly significant relationship between the amount of oestradiol in saliva to that not bound to carrier-protein in blood (Fig. 7; $r = 0.78$; $P < 0.001$). The slope of this linear regression was 0.34 which implies that the amount of salivary oestradiol was appreciably less than that found free in blood.

DISCUSSION

Levels of salivary oestradiol in normal parous women throughout the menstrual cycle were virtually identical to those in patients with early breast cancer who were still maintaining regular menstrual cycles after treatment. The pattern of salivary oestradiol levels over the menstrual cycle in which there is a peak in the late follicular phase followed by a second rise in the luteal phase is

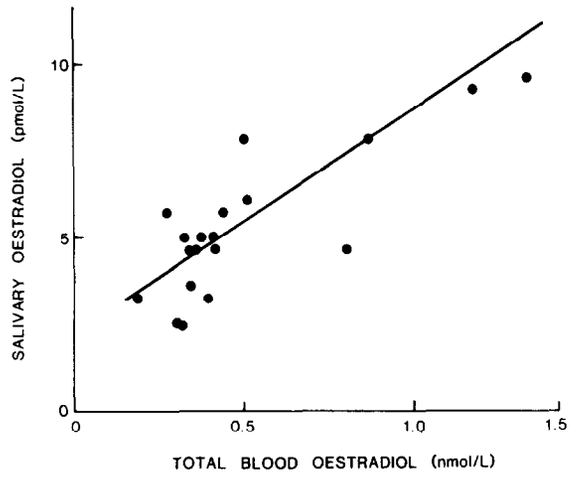

Figure 6. Relationship between salivary and total blood oestradiol. The equation of the linear regression of oestradiol levels in saliva and blood in 19 volunteers without breast cancer was y = 6.5x + 2.34 *where* y *is the amount of salivary oestradiol* (pmol/l) *and* x = *total oestradiol* (nmol/l). *The interval of time between collecting saliva and blood specimens was less than* 15 min.

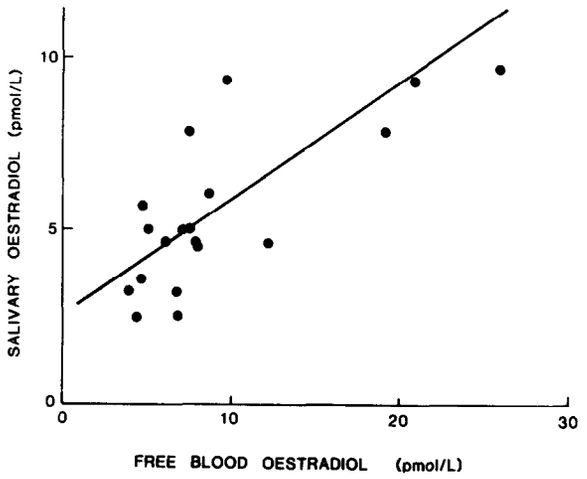

Figure 7. Relationship between salivary and free blood oestradiol. The equation of the linear regression of oestradiol levels in saliva against non-protein bound blood oestradiol is y = 0.34x + 2.65 *where* y *is the amount of salivary oestradiol* (pmol/L) *and* x *is the amount of non-protein bound oestradiol* (pmol/L). *The* 19 *normal women are the same as those described in Fig.* 6.

similar to the well-documented changes which occur in blood. However, these changes in salivary levels are noticeably less pronounced that those which occur in blood. The mid-peak of salivary oestradiol which occurred at day 11 is in accord with the data of Donaldson *et al.* [18] who reported a peak at day 12 (assuming the LH peak occurred on day 14). The present mid-cycle peak value of 10 pmol/l is similar to 8 pmol/l reported by Donaldson [18] but less than 22 pmol/l and 50 pmol/l reported by Evans *et al.* [19] and Walker *et al.* [20], respectively. The second oestradiol peak during the luteal phase of 7–8 pmol/l lies within the lower range of 4 pmol/l, 22 pmol/l, 25 pmol/l and 29–129 pmol/l reported by Donaldson *et al.* [18], Evans *et al.* [19], Walker *et al.* [20] and Choe *et al.* [21], respectively.

Patients with breast cancer had normal levels of salivary progesterone during the menstrual cycle which is in accord with the recent report of Read and his colleagues [12]. The single luteal phase peak of salivary progesterone is similar to that found to occur for blood progesterone. The present peak value of about 350 pmol/l falls in the range 300–500 pmol/l reported by others [9, 22–24].

The saliva samples in this study were collected between 9 a.m. and 9 p.m., the time being determined by what was convenient for the volunteer. After the commencement of this study it was reported by Read *et al.* [12] that the salivary progesterone levels were higher in the evenings than the mornings. However, for the following reasons we do not believe that this factor alters the conclusions of this study.

Firstly, the difference was small and only significant if statistically analysed within subjects. Certainly in this study the time of collection did not significantly influence salivary progesterone levels when analysed on a between-subject basis. The coefficient of variation between subjects in the study of Read *et al.* [12] was of the order of 50% for both morning and evening progesterone levels. Thus the added variance due to not controlling for time of collection would be trivial compared with the overall between-subject variance. Secondly, in the study of Read *et al.* [12] the morning–evening difference was only significant in control women but not for those patients with breast cancer. Thirdly, the conclusion that there is no significant difference between the concentration of salivary progesterone in patients with breast cancer and controls is the same as that arrived at by Read *et al.* [12] who controlled for time of saliva collection.

There are several reports claiming that the amount or proportion of free oestradiol is raised in women who have either breast cancer or an increased risk of developing the disease [2–7]. One of the reasons for the present investigation was to determine whether the amount of salivary steroid is equivalent to the levels of biologically available steroid in blood as has been claimed [8–10]. It is therefore surprising that, in the light of so much evidence implicating blood free oestradiol in the aetiology of breast cancer, no abnormalities could be discerned in salivary oestradiol levels. Since our results are contrary to what might have been expected we examined whether salivary oestradiol levels are equal to the amount of free oestradiol in blood. Despite there being a highly significant linear correlation between these two parameters,

the amount of salivary oestradiol was less than half that for serum free oestradiol. Chearskul *et al.* [25] also found significantly less oestradiol in saliva than the free steroid in blood. For salivary and serum-free oestradiol to be equal in concentration, would require that only 1% of oestradiol in blood is free; a situation only normally found in pregnancy [17,26]. The mean percentage (± S.D.) of free oestradiol in sera was found to be 1.84% (± 0.29%), which agrees with 2.2% by equilibrium dialysis [27] and 1.6% by centrifugal ultrafiltration [17]. Evans and his colleagues [19] reported that the concentration of oestradiol in saliva was 0.1–0.2% of levels found in plasma.

In addition to the present results for oestradiol there are other steroids for which the amount found in saliva does not appear to be equivalent to that found free in blood. Thus the conversion of cortisol to cortisone in the salivary gland [28] makes the interpretation of salivary cortisol levels uncertain. Studies of this hormone by Uemeda *et al.* [29] led them to conclude that the passage of steroid from the blood stream to parotid fluid involved more than simple diffusion. Baxendale and James [30] found that in women there was about three times as much testosterone in saliva as free in plasma and ten times as much in the case of 5α-dihydrotestosterone. Salivary progesterone is also only one fifth of that found free in serum [15]. It is also debatable whether only the free amount of steroid, as measured by dialysis, is biologically available since it has been claimed that appreciable amounts of steroid bound to albumin are also readily available to target tissues. Thus in British women about 70% of blood oestradiol is bound to albumin [7] and Pardridge and Landlaw [31] claim that as much as 50% of this oestradiol is extracted across the blood–brain barrier.

Although some women found the notion of collecting saliva samples aesthetically unacceptable there is no doubt that salivary assays are useful for monitoring changes in steroid hormone levels in some circumstances and that they offer advantages over blood assays (see [10]). However, it is clear from the present results that the measurement of salivary oestradiol and progesterone has shed no light on the involvement of ovarian hormone secretion in the aetiology of breast cancer. Furthermore, the present data on salivary oestradiol appear to be at variance with those obtained in other studies on non-protein-bound steroid in blood of women with breast cancer.

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