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Evaluation of Luteal-phase Salivary Progesterone Levels in Women with Benign Breast Disease or Primary Breast Cancer*

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Abstract—*Salivary progesterone concentrations were determined in premenopausal parous women with a mean age of* ca. 40 yr *who had a history of either benign breast disease* (n = 15) *or primary breast cancer* (n = 15) *and in a group of age-matched healthy women* (n = 15). *Saliva samples were collected at* 09.00 *and* 21.00 hr *daily for one complete menstrual cycle and progesterone concentration was measured by radioimmunoassay. Characteristic luteal-phase progesterone profiles were observed in all subjects in each of the three groups but no statistical inter-group differences could be demonstrated for age-matched subjects in each group. These studies indicated that ovarian dysfunction, as judged from salivary progesterone concentrations, was not apparent in older premenopausal women with a history of benign breast disease or primary breast cancer when compared with age-matched controls.*

INTRODUCTION

DESPITE intensive research over the past two decades [1], the role of the various hormones in the aetiology of breast cancer remains to be elucidated. It appears reasonably clear to most that the ovarian steroid hormones oestradiol-17β and progesterone are implicated; for example, early premenopausal oophorectomy has been shown to reduce the risk of breast cancer [2]. Furthermore, early menarche [3, 4] and late menopause [3, 5], which increase the effective reproduction life of the female, have been reported to increase the risk. This is presumably related to the number of menstrual cycles, and the changing concentrations of female sex hormones to which the maturing and aging breast is subjected [6–8]. These risk factors [9], together with the now well-accepted protective effect of early first-term birth [10], suggest that the ovarian steroid hormones are concerned in the aetiology of the disease between the time when a female goes through puberty and the first full-term pregnancy, when it is considered a breast is 'biologically' mature [7]. In a hypothesis propounded by Sherman and Korenman [6] it has been suggested that this period of time be considered the 'first window', in which abnormal steroid patterns during menstrual cycles may be concerned in the promotion of early carcinogenesis. The 'second window' is related to the 5- to 10-yr period prior to the menopause, when abnormal cycles are also frequently observed. Of the two female sex hormones produced during the cycle, oestradiol has generally been considered growth-promoting whereas progesterone, based on accumulated data derived from studies of the uterus, has been thought to exert some protective effect against overstimulation by the oestrogen. High or normal levels of oestrogen during the cycle, in the presence of low progesterone resulting from abnormal luteal function, could therefore be considered a risk factor. On the other hand, there is evidence that progesterone itself may be mitogenic in breast tissue under certain conditions [11, 12] and it is possible that this hormone may promote the growth of early breast cancer.

This hypothetical hormonal imbalance may not persist until the disease is clinically manifest. Mauvais-Jarvis and his colleagues [13], however, evaluated ovarian function by measuring plasma oestradiol and progesterone during the luteal

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phase of the cycle in 109 patients with various mastopathies and in 50 women exhibiting 'normal' ovulatory cycles. Data from these studies indicated that in benign breast disease there was a reduction in plasma progesterone, and in those patients with fibroadenomas and increased nodularity of the breasts oestradiol was elevated. These findings [13], together with the possibility that women whose infertility was thought to be due to luteal-phase insufficiency may be more likely to develop breast cancer [14] and the fact that benign breast disease is a risk factor for breast cancer [15] prompted a detailed study of salivary progesterone levels in premenopausal women with a history of benign breast disease or primary breast cancer and a group of age-matched controls.

MATERIALS AND METHODS

Subjects

These consisted of three groups of parous, premenopausal women ($n = 3 \times 15$) residing in the Glasgow area. Group I comprises 15 women who had had a Patey mastectomy for primary breast cancer at least 6 months prior to the commencement of the study. All these patients were judged clinically to be disease-free and none had current breast pain or had received radiotherapy or other adjuvant therapy. Group II contained those who had been treated for benign breast disease by lumpectomy, again at least 6 months previously, and in whom the breast pathology had shown evidence of epitheliosis. Group III was a control population of clinically healthy women. Individuals in all three groups were matched across groups for age and body weight. The protocol called for the exclusion of subjects who had been treated by endocrine surgery other than that cited; those on oral contraceptive or hormone medications of any kind within the past year; those judged to be obese and those engaged in shift-work or existing on a general wake-span outside the limits of 07.00 to 00.00 hr.

Group I women were selected from the records at the Western Infirmary. Firstly, all women under the age of 50 yr were identified and constituted very approximately 25% of the total. Further selection for age-matching and compliance with exclusion criteria meant that the final 15 subjects represented 20% of the patients under 50 yr of age. No women would have been excluded on the bases of short or long cycles, or on the basis of problematic early menstrual years or because of complaints about their cycles; these situations were not encountered.

All subjects were interrogated using a questionnaire primarily concerned with their reproductive life history. Table 1 displays group statistics relevant to the study. These data in tabular form implicitly indicate the degree of matching between groups for age and body weight, and the comparability of some of the reproductive factors of importance. Explicitly, however, subjects were age-matched across groups, generally within 2 yr. It is emphasized that no subjects professed to oral contraceptive use within 1 yr prior to the commencement of the study. In all groups some subjects had used oral contraceptives sometime in the past: details of use are documented in Table 1. Those who successfully breast fed one or more children numbered eight in the control group, two in the epitheliosis group and six in the primary cancer group; those who failed to breast feed numbered one, two and two respectively in these groups; otherwise subjects chose not to breast feed. The mean ages in years at surgical treatment were 37.1 (5.7; 27–45) and 37.2 (4.9; 28–43) for the benign and primary breast cancer group respectively; standard deviation and range are given in parentheses. The mean times since operation for the benign and cancer groups were both 2.7 (1.6; 0.5–5 yr) with standard deviation and range given in parentheses.

Samples

Subjects were supplied with an instruction/record card and two compact plastic boxes each containing an insert for holding up to 40 5-ml glass sample tubes. Samples of whole saliva were taken at 09.00 and 21.00 hr daily for one complete menstrual cycle. Following collection, samples were immediately placed in a plastic box and transferred to the freezing compartment of the subject's domestic refrigerator. These refrigerated containers were collected by the field investigator, transported to the Western Infirmary, Glasgow, and stored at −20°C. Samples were despatched by special courier to the Tenovus Institute, Cardiff, where they were stored with quality control samples containing comparable amounts of progesterone to monitor storage stability. Matched plasma and saliva samples were taken from each subject on at least one occasion during the 'luteal' phase of the cycle.

Coding of samples

Subject samples were coded by the Glasgow team so that the Tenovus investigators were only aware of the existence of three groups, designated as A, B and C. At the completion of the analysis, the results were despatched to Glasgow and only then were code identities revealed.

Table 1. Pertinent clinical details for clinically healthy parous premenopausal women (controls) and comparable women with a history of benign breast disease or primary breast cancer

Clinical details	Group Control ($n = 15$)	Benign ($n = 15$)	Cancer ($n = 15$)
Age (yr)			
Mean	41.1	39.8	39.9
S.D.	4.6	4.9	5.1
Range	33–49	30–46	31–45
Weight (kg)			
Mean	57.6	59.1	57.6
S.D.	6.6	5.8	6.0
Range	49.0–72.3	50.9–65.5	48.6–66.8
Age of menarche (yr)			
Mean	12.6	13.1	13.1
S.D.	1.3	1.7	1.9
Range	11–15	11–18	10–16
Cycle length (days)			
Mean	25.9	25.9	28.0
S.D.	1.5	2.6	3.3
Range	23–28	23–30	24–37*
Age at 1st pregnancy (yr)			
Mean	25.9	25.7	23.9
S.D.	4.1	4.8	3.9
Range	20–35	18–37	19–31
No. of pregnancies			
Mean	3.1	2.3	2.5
Range	1–5	1–4	1–5
History of oral contraceptive use	4 N; 3 LS; 7 GS	5 N; 6 LS; 4 GS	4 N; 2 LS; 9 GS
No. of subjects with familial history of breast cancer	zero by definition	2	6

N, LS and GS refer to the number of women who have never (N) used oral contraceptives or those whose use has been less than (LS) or greater than (GS) 6 months.

*Exclusion of the two highest values gave a range of 24–29 days.

Assay design

The arrangement of standards, and subject and quality control samples within an assay batch has been previously described [16], together with the techniques used for RIA data processing and internal quality control. The arrangement of the subject samples was such that each batch, which was of limited size, contained six patient samples from each of the three groups for the same day of the cycle. This design served to reduce inter-assay variation effects and tended to maximise any inter-group differences that may exist between progesterone values.

Assay of progesterone in saliva and plasma

The procedure for measuring salivary progesterone has been published elsewhere [17]. Briefly, the method consisted of thawing the samples, centrifuging at 700 *g* to remove particulate matter and extracting duplicate 400-μl aliquots of saliva with 3 ml of petroleum ether. The extraction tubes (100 × 12 mm, glass) were placed in an acetone/CO_2 mixture, the aqueous phase frozen and the organic phase removed and evaporated to dryness under nitrogen. The residue was dissolved in 100 μl of progesterone antiserum (raised in rabbits to a progesterone-11α-hemisuccinate/bovine serum albumin conjugate; 1 in 8000 dilution with phosphate buffer), incubated at 30°C for 1 hr and 100 μl of tritiated label (16,200 dpm) added and further incubated for the same period. The radioactivity associated with the bound fraction was measured after separation of free from bound progesterone, using dextran-coated charcoal. The dose was determined from the calibration curve of bound radioactivity for 10-μl aliquots of progesterone standards, in ethanol, containing 1–10 μg/l. Plasma progesterone was assayed using a commercial kit (Diagnostic Products Corporation, Los Angeles, CA) which utilized a ^{125}I-radioligand and antibody-coated tubes.

Statistical analysis

Quality control. Each assay batch was monitored for changes in the mean, imprecision and drift in progesterone values for low-, medium- and high-titre quality-control pools as previously described [16].

Characterization of individual cycles. A method has been devised in this institute for the characterization of progesterone profiles. This method has been assessed for its accurate identification of the time of ovulation using matched saliva and plasma samples; plasma progesterone and luteinizing hormone, salivary progesterone and basal body temperature provided pertinent information in a study of clinically healthy premenopausal women. Based on salivary progesterone measurements from samples collected daily at 09.00 hr through the menstrual cycle, the accuracy of identifying the time of ovulation was judged to be generally within 1 day [Walker *et al.* submitted for publication]. The salivary progesterone method has also been supported by ultrasound studies, albeit on only a few patients to date. The statistical method consisted of two parts: the first calculated the number of follicular phase samples that were used to determine the mean basal follicular progesterone value and consequently the day of progesterone rise, i.e. 'ovulation', and the second part of the method characterized the 'luteal'-phase profile in the form of a cumulative frequency distribution. The computer program, written in BASIC, is available from D.W.W. on request, and the principle of the method is shown in Fig. 1. On a point of definition, the term 'moving average' is synonymous with 'updated average'. The 'moving' element refers to the addition of data for consecutive days of the cycle to all previous data, thereby creating successive (moving) means.

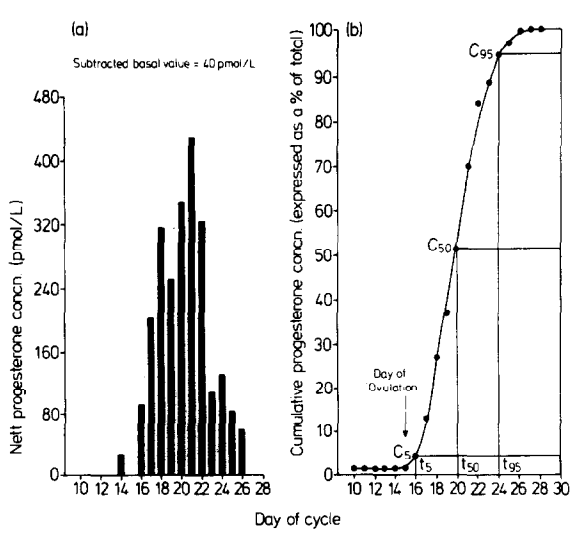

Fig. 1. Numerical characterization of menstrual cycle profiles: (a) nett salivary progesterone output during the luteal phase of the cycle and (b) the cumulative frequency characteristics of the nett progesterone distribution.

The basal follicular progesterone value was obtained initially by performing a moving average along the 'follicular' values until a situation was reached when three consecutive values ahead of the current datum were 1.4 times the current moving average. The value of 1.4 was an empirically derived constant obtained from experience in the above-mentioned study. The initial 'pass' of the data provided the first stopping rule (i.e. the non-incorporation of 'luteal' progesterone into the basal value) and so determined the number of samples judged to be follicular in origin, and hence the mean and standard deviation of the basal value. This standard deviation was then used to reject outliers outside ±2 S.D.s of the mean. The moving-average process was repeated to provide a new mean basal value and associated standard deviation. Finally, the third 'pass' involved scanning sequentially through the data to find the day when three consecutive days ahead of the current datum were greater than the second estimate of the mean basal value plus its 2 S.D.s. The current point which satisfied these criteria provided the day of 'ovulation' and the final number of follicular phase samples that can be used to calculate the last estimate of the mean basal progesterone value.

The characterization of luteal-phase progesterone profiles was achieved by subtracting the basal value from all progesterone measurements, and performing calculations to derive the cumulative frequency distribution (i.e. if the basal value were looked upon as a target mean then this calculation is equivalent to a cumulative sum procedure). The same number of daily samples was measured for each individual in each group. Figure 1 illustrates the principle of calculating and characterizing cumulated progesterone levels at the 5, 50 and 95% levels together with their corresponding times.

Statistical tests. Comparison of data from age-matched pairs of subjects has been achieved using the paired Student's *t* and Wilcoxon tests, and correlations were based on the Pearson product moment.

RESULTS

Progesterone levels were measured in daily 09.00 and 21.00 hr samples for each group investigated from the second half of the follicular phase to the end of the cycle. The means and standards errors of the means for the 09.00 and 21.00 hr samples are shown in Fig. 2, and

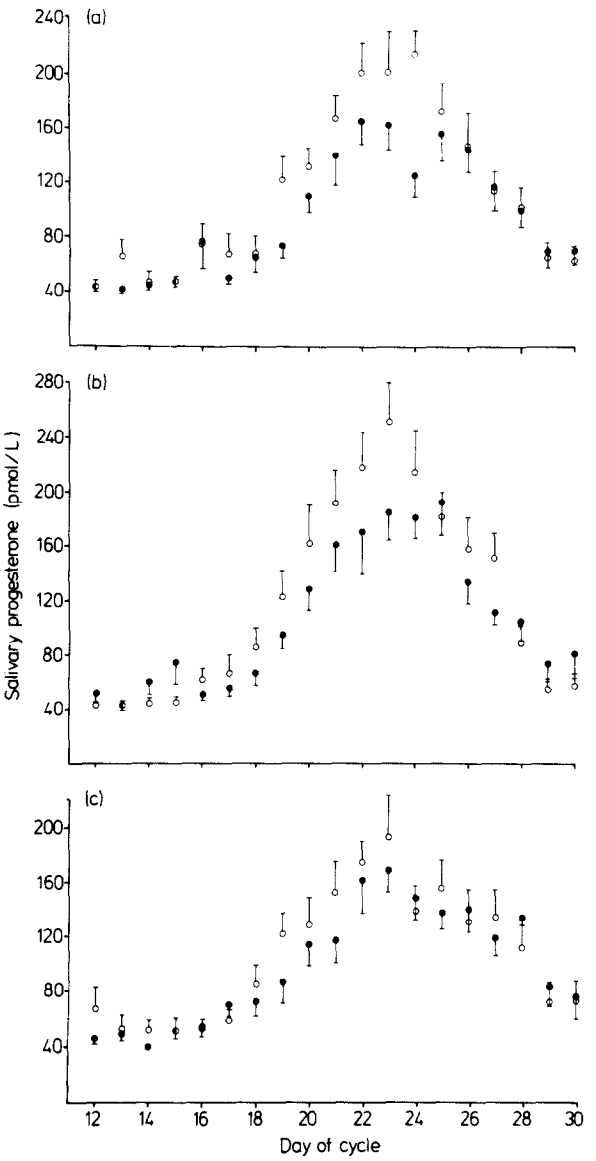

Fig. 2. Comparison of mean salivary menstrual cycle progesterone profiles and associated uncertainties (S.E.M.) for: (a) controls, (b) benign and (c) primary breast cancer groups of parous premenopausal women (09.00 hr samples, ♀; 21.00 hr samples, ♀).

composite diagrams of the means without error bars for both a.m. and p.m. samples are presented in Fig. 3. The distribution of progesterone means and their standard errors for comparable days of the cycle for each group of subjects were almost identical within either the morning or evening samples. Comparison of the mean morning and evening progesterone profiles for the luteal phase of the cycle (Fig. 3) indicated that progesterone values for each group were elevated in the evening, perhaps to a lesser extent in the cancer group, the shape of the evening distributions being more leptokurtic. Since this manner of data presentation can conceal important features associated with individual distributions, progesterone profiles for each subject within a group

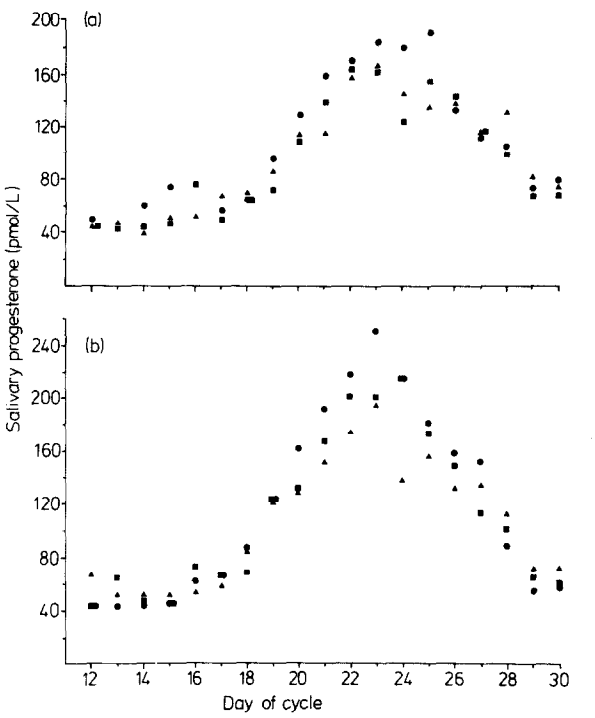

Fig. 3. Comparison of mean menstrual cycle salivary progesterone profiles for control (■), benign (●) and primary cancer (▲) groups of parous premenopausal women, for: (a) 09.00 hr and (b) 21.00 hr samples respectively. Data are presented from Fig. 2 in compacted form.

were analyzed using the cumulative statistics previously describd in the Materials and Methods section. The intra- and inter-assay variation expressed as % coefficient of variation of the mean value of progesterone in each of the low-, medium- and high-titre pools for various periods are shown in Table 2. These periods of analysis are consecutive and approximately equi-spaced in 1-month intervals. Data indicate reasonable intra-assay variation but the inter-assay variation is somewhat higher than expected. The mean progesterone value for each pool and for each period also show variation a little higher than that experienced in other studies, despite the rigorous attention paid to the problem [16]. These data support the statistical design used in the analysis and, *inter alia*, the ensuing parametric and non-parametric 'matched-pair' statistics (paired Student's t and Wilcoxon tests) used to analyse the data.

Profile analysis provided values for three parameters, C_5, C_{50} and C_{95}, which are the cumulative progesterone values for 5, 50 and 95% of the total nett progesterone concentration obtained for each individual (Fig. 1) for either the morning or evening samples collected through the menstrual cycle. The days of the menstrual cycle equivalent of C_5, C_{50} and C_{95} were also recorded. These data are shown in Table 3, where

Table 2. Intra- and inter-assay variation for the salivary progesterone assay over a period of 6 months; *each consecutive month is identified by periods* 1–6 *respectively*

Period of analysis	No. of batches	Mean assay variation (% C.V.) Intra-batch L	M	H	Inter-batch L	M	H
		Morning samples					
1	18	14.1	7.4	9.2	17.1 (63.3)	10.4 (294.9)	8.7 (476.3)
2	18	11.7	11.1	5.9	15.1 (81.3)	27.0 (259.6)	16.0 (611.0)
3	9	9.6	6.2	8.1	15.7 (77.4)	18.8 (295.1)	16.7 (598.9)
		Evening samples					
4	18	11.0	12.6	9.5	15.0 (83.0)	25.2 (242.6)	12.6 (566.9)
5	18	9.2	8.5	5.9	20.2 (87.7)	17.6 (231.7)	12.2 (617.1)
6	9	12.0	8.6	5.4	15.0 (93.6)	9.6 (230.5)	8.5 (647.9)

Data were obtained from low (L)-, medium (M)-, and high (H)-titre internal quality control pools. The mean progesterone concentrations (pmol/l) are given in parentheses.

Table 3. Cumulative salivary progesterone values at the C_5, C_{50} *and* C_{95} *levels together with their respective times measured in menstrual cycle days (presented as means and standard deviations for the control, benign and primary breast cancer groups of parous premenopausal women)*

Progesterone metameter	Sampling Time	Progesterone concentration Control Mean	S.D.	Benign Mean	S.D.	Cancer Mean	S.D.	Day of menstrual cycle Control Mean	S.D.	Benign Mean	S.D.	Cancer Mean	S.D.
C_5	M	37.2	18.1	49.8	26.5	56.6	28.8	16.8	2.2	16.5	3.0	17.9	2.4
	E	62.8	24.5	61.7	33.9	60.0	42.7	16.8	2.0	16.7	2.6	15.4	2.6
C_{50}	M	541	222	556	238	503	259	22.4	2.0	22.1	1.9	22.5	2.2
	E	678	199	728	281	686	425	21.5	1.2	22.1	1.6	21.8	1.6
C_{95}	M	989	385	1097	426	1008	501	26.9	2.1	26.9	1.8	27.1	1.7
	E	1278	385	1398	552	1360	814	26.3	1.5	26.4	1.6	26.9	1.9
Basal value	M	44.5	4.6	46.8	5.9	46.9	6.8	–	–	–	–	–	–
	E	51.3	17.9	42.8	2.6	52.6	16	–	–	–	–	–	–

M and E represent 09.00 and 21.00 hr samples respectively.

the cycle metameters are expressed as means and standard deivations.

No significant differences exist between basal levels of progesterone for each of the three groups, which means that no pronounced bias was introduced into the calculation of nett progesterone concentration. This is important since a moderate bias, cumulated over several days, would markedly change the values of C_5, C_{50} and C_{95}. Comparison of values for the C_5, C_{50} and C_{95} cumulative progesterone values for each group using both the paired t test and the Wilcoxon test for both morning and evening samples failed to reveal statistically significant differences at the $P<0.05$ level. It is emphasized that subjects were not only paired for age between groups but samples taken on the same day of the cycle were also analysed in the same batch of assays; thus the effect of any systematic drift in the assay was reduced. Significant differences using the paired t test and Wilcoxon test were observed between the morning and evening C_{50} and C_{95} values ($P<0.002$ for all tests) for both control and benign groups but in the cancer group this was not the case ($P>0.05$). As shown in Table 4, differences in the time of progesterone rise, indicative of ovulation, measured in days from the onset of the next menses, were not statistically significant between any of the three groups; neither were the lengths of the first half of the luteal phase (t_{50}–t_5), the lengths of the second half of the luteal phase (t_{95}–t_{50}), the lengths of the luteal phase corresponding to the 90% interval (t_{95}–t_5) nor the length of the luteal phase (t_{95}–time of ovulation) statistically significant. No significant correlation of luteal-phase characteristics with age was demonstrated

Table 4. Calculated times of ovulation, duration of first half (t_{50}–t_5), second half (t_{95}–t_{50}) and 'total' (t_{95}–t_5) width of the luteal phase of the cycle for the control, benign and primary breast cancer groups of parous premenopausal women

Menstrual cycle metameter	Sampling time	Control Mean	Control S.D.	Benign Mean	Benign S.D.	Cancer Mean	Cancer S.D.
Day of progesterone rise (ovulation)	M	12.2	1.2	11.7	1.7	11.1	2.9
	E	12.2	1.6	12.5	3.0	11.5	2.5
t_{50}–t_5	M	5.9	2.1	5.8	2.9	4.6	1.5
	E	5.6	2.3	5.5	2.9	6.4	3.4
t_{95}–t_{50}	M	4.7	1.1	5.1	1.2	4.6	1.0
	E	4.9	1.3	4.5	1.4	5.1	1.6
t_{95}–t_5	M	10.6	3.0	10.9	3.2	9.2	1.8
	E	10.5	3.2	10.0	3.2	11.5	3.4

M and E represent 09.00 and 21.00 hr saliva samples respectively.

in any of the three groups at the 5% level. It was concluded that there were no statistically significant differences in progesterone profiles among the three groups of premenopausal women studied, which indicated comparable ovarian function.

Finally, comparison of the correlation coefficients of matched plasma and saliva samples for all three groups gave almost identical correlations of $r = 0.90$ ($P < 0.001$), indicating the close relationship between the relative concentrations of progesterone for both media.

DISCUSSION

Mauvais-Jarvis and colleagues [13, 18] reported that plasma progesterone levels, measured during the luteal phase of the menstrual cycle, were lower in women with mastodynia, and in those with both mastodynia and cystic mastitis or fibroadenomas, when compared with a 'normal' premenopausal group. The conclusion drawn from this study was that women with benign breast disease and mastodynia have inadequate corpus luteum function. Furthermore, the fact that the use of oral contraceptives with a higher progestagen to oestrogen ratio appeared to reduce the incidence of benign breast disease [19, 20] is of interest. Counter-arguments can, however, suggest an antithesis to the Sherman and Korenman hypothesis, in that prolonged exposure to progesterone might well be a risk factor for breast cancer. Supporting tentative evidence for this antithesis is provided by the report [21] that girls reaching menarche at an early age settle down to normal ovulatory cycles quicker than those girls who have a later age of menarche. Using this hypothesis, therefore, early menarche. which is a risk factor for breast cancer, would result in a longer exposure of the breast to progesterone. In a recent publication [22] it was suggested that a 'high' progestagen content of oral contraceptive used by women before the age of 25 led to a substantial increased risk of breast cancer. Pills containing a low dose of progestagen were said to have little or no effect on risk. Masters and colleagues [11] identified cyclic variation of DNA synthesis in breast epithelium during the menstrual cycle. These studies and data of mitotic activity, also in breast epithelium [12], demonstrated peak activity in the luteal phase, again implicating progesterone in the aetiology of breast cancer. Clearly, an investigation of luteal phase progesterone secretion in clinically healthy individuals, and in those subjects who have been treated for benign breast disease or primary breast cancer, is important: thus correlating the degree of ovarian dysfunction (if it exists) with the types of disease described. The choice of saliva is preferred since the progesterone content represents the 'biologically' active, non-protein-bound concentration found in plasma; it is a stress-free procedure facilitating multiple sampling, and is easily performed by an individual, and samples can be conveniently stored in the domestic refrigerator [16, 23]. In contrast, urinary pregnanediol depends not only on ovarian secretion but also upon hepatic and renal function [24], whereas the choice of blood samples is limited by the number that can be collected. The choice of a single sample in the thermal plateau of the luteal phase of the cycle or even a mean of three samples [13] is affected by both luteal and circadian variations. Daily morning and evening saliva samples reduce the relative magnitude of biological uncertainty arising from variation in progesterone concentration during the day, and, as previously discussed, there is good correlation between saliva and plasma hormone for all three groups.

The present study of approximately 1800 samples, obtained from a total of 45 subjects, failed to reveal any statistically significant differences between progesterone concentrations for the control group and for subjects in the benign and

cancer categories. Inter-group comparisons utilized an analytical design to minimise the effects of inter-assay variation, and used paired parametric and non-parametric statistics on age-matched subjects from each group in the overall analysis. In addition to simply evaluating data in the manner expressed in Figs 2 and 3, ascendent and decendent characteristics of progesterone secretion were investigated for both halves of the luteal phase. This was achieved using the properties of the cumulative frequency distribution for both morning and evening progesterone concentrations collected daily during the menstrual cycle. Statistical significance between groups for any of these cumulative frequency metameters was not demonstrated. It was possible that older women may have a different data structure to those of younger women. Analysis of luteal progesterone cumulative frequency metameters between groups, for those over 41 yr ($n = 3 \times 7$), were compared, but again no statistically significant findings emerged. This was also true of the younger group of women under 42 yr of age ($n = 3 \times 8$). Using cumulative progesterone concentrations at the 50% (C_{50}) and 95% (C_{95}) levels, morning and the higher evening values were significantly different ($P < 0.002$) within both control and benign groups but this was not so for the cancer group. This study, however, was not set up to test for systematic circadian variation and, although a trend to higher evening values was observed in control and benign groups, this finding should be re-assessed using a different analytical design. Clearly, differences in circadian, between-cycle and seasonal variations in salivary progesterone in subjects for each group has still not been exhaustively investigated. Preliminary data on these sources of variation, obtained from clinically healthy premenopausal women, are published elsewhere [25]. In conclusion, it would seem that luteal dysfunction, as judged from salivary progesterone measurements in parous and mature premenopausal women, is not in evidence in women with a previous history of benign breast disease or primary breast cancer.

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