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Post-suckling prolactin:oestradiol ratio—a potential index to predict the duration of lactational amenorrhoea in women

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To assess whether the duration of lactational amenorrhoea can be predicted in individual women, we studied the pre- and post-suckling concentrations of immune prolactin (IR-PRL) and of bioactive prolactin (BIO-PRL) and basal concentrations of oestradiol in ten amenorrhoeic fully nursing women at 3 months post-partum. The women were of similar age, weight and had infants of similar growth rate. Five of these women were to experience long amenorrhoea (>180 days) and the others short amenorrhoea (<180 days). Blood samples were drawn 30 min after a suckling episode initiated at 0800 h, 1600 h and 2400 h. BIO-PRL distinguished between groups of women at 0030 h but not at other times, while there was considerable overlap between values for IR-PRL and oestradiol at all times studied. At 1630 h, the ratios post-suckling BIO-PRL: oestradiol and post-suckling IR-PRL:oestradiol were above 2000 in the women that were to experience long amenorrhoea and below this threshold in the other women. The ratio post-suckling BIO-PRL:oestradiol provided more information since the difference between the lowest ratio in the long amenorrhoea and the highest ratio in the short was 699, while it was 520 for the IR-PRL:oestradiol ratio. The determination of these ratios may help to predict the duration of lactational amenorrhoea in individual fully nursing women.

Key words: lactational amenorrhoea/oestradiol/prolactin

Introduction

Lactation provides a natural source of nutrients for the infant and determines a period of infertility in the mother that allows her to space interbirth intervals. Duration of lactational amenorrhoea is heavily dependent upon exclusive breast-feeding. Introduction of supplementary feeds decreases the duration of amenorrhoea (Howie *et al.*, 1981; Tay *et al.*, 1996). However, when women are fully or nearly fully breast-feeding, lactation affords 98% protection from pregnancy in the first 6 months post-partum, providing the basis of the lactational amenorrhoea method (LAM) (Bellagio Consensus Statement, 1988; Kennedy *et al.*, 1989; Díaz *et al.*, 1991a; Short *et al.*, 1991; Kennedy and Visness, 1992; Pérez *et al.*, 1992). Although LAM is very effective as a contraceptive method, many fully nursing women and their physicians are sceptical about its use and resort to other contraceptive methods, even if unnecessary. To identify whether an individual breast-feeding woman will recover fertility before or after 6 months post-partum will allow her to time the initiation of contraception accordingly.

In fully breast-feeding women, at the first months post-partum two hormones, prolactin and oestradiol, show differences in plasma concentration that are associated with the duration of lactational amenorrhoea (Díaz *et al.*, 1989; Díaz *et al.*, 1991b; Campino *et al.*, 1994a). The differences precede the recovery of ovarian function by 1 to 3 months. Suckling induced a greater release of immunoreactive prolactin (IR-PRL) and bioactive prolactin (BIO-PRL) in women who were going to experience long amenorrhoea (>6 months) than those who were to experience short amenorrhoea (<6 months) (Díaz *et al.*, 1989; Campino *et al.*, 1994a). Prolactin response to suckling reached its maximum 30 min after the initiation of a suckling episode. This response was minimal at 0830 h, and greater at evening and night times. In addition, the mathematical relationship between BIO- and IR-PRL differed in both groups after suckling, since the same amount of IR-PRL corresponded to a higher concentration of BIO-PRL in the women who were to experience long amenorrhoea than in those who were to experience short amenorrhoea (Campino *et al.*, 1994a). These observations suggest qualitative differences in the response to suckling that improve biological activity of PRL in the long amenorrhoea women.

Oestradiol concentration also showed differences in these nursing women. The mean oestradiol concentration was significantly lower in the group of nursing women who were to experience long amenorrhoea than in the group who were to experience short amenorrhoea (Díaz *et al.*, 1991b).

In the light of these findings, we analysed the former data to test whether prolactin and oestradiol concentrations in individual amenorrhoeic nursing women at 3 months post-partum could indicate the recovery of ovarian function before or after 6 months post-partum. Our results suggest that the ratio post-suckling prolactin:oestradiol could serve such a purpose.

Materials and methods

Subjects

The original study was performed around day 90 post-partum in ten amenorrhoeic healthy nursing women who were fully breast-feeding and whose infants had a normal growth rate. The experimental procedure was explained to the women, who gave their consent in

writing. Volunteers were chosen at the time of delivery among healthy women who wanted to breast-feed their child for as long as possible. They were 20–30 years old, parity 1–3, and had a normal pregnancy ending at 38–40 weeks' gestation in vaginal delivery of a healthy child of normal weight. They were not using therapeutic drugs or hormonal contraception. The women were instructed not to feed their babies any liquid or solid food or water and to use their breast as the only source of water and nutrients during the first 6 months post-partum, except for the administration of vitamin drops. They were followed for 12 months at the clinic recording the exact date of their first post-partum menses. Five of the women recovered their cycles between 5 and 6 months post-partum (short amenorrhoea group). The other five women remained in amenorrhoea for more than 6 months (range 8–10 months, long amenorrhoea group). Subjects were part of the previous studies on IR-PRL (Díaz *et al.*, 1989b) and BIO-PRL (Campino *et al.*, 1994a). A detailed description of the selection and follow up procedure is found in these papers.

Blood sampling protocol

To perform the study, the women and their infants were hospitalized for 48 h in the metabolic unit at the Hospital Clínico de la Pontificia, Universidad Católica de Chile. Standardized meals were provided at fixed times. The women remained sitting or lying down for the major part of the day, took care of their infants assisted by a nurse and continued their usual breast-feeding practices while in the unit. The time and duration of each nursing episode were recorded during the 24 h sampling period. The first 24 h were allowed for adaptation to the environment and a butterfly needle was inserted into an antecubital vein between 0630–0715 h on the second day. Blood samples were collected at 2 h intervals, starting at 0800 h and ending at the same time the following day. In addition, to study the effect of suckling, blood was collected at 10 and 30 min after the initiation of the suckling episodes, which occurred at approximately 0800, 1200, 1600, 2000, 2400 and 0400 h. In each woman the sampling schedule was adjusted to the breast-feeding pattern in order to obtain the basal samples at least 90 min after the end of the preceding nursing episode. Nursing episodes were defined as events in which the infant suckled one or both breasts, separated by at least 30 min from the preceding and following episodes. A suckling contact was defined as the period during which the baby suckled one breast continuously, once or several times within each nursing episode (Díaz *et al.*, 1989b). Five millilitres of blood were obtained in tubes containing heparin at the times indicated above. The blood samples were centrifuged and the plasma was stored –20°C until assayed.

Laboratory assays

BIO-PRL was measured by the Nb2 lymphoma cell assay, IR-PRL and oestradiol were measured using the reagents and methodology of the WHO Program for the Provision of Matched Assay Reagents for the RIA of Hormones in Reproductive Physiology. The details of these methods were described in a previous study (Díaz *et al.*, 1991b; Campino *et al.*, 1994a). Stability of BIO-PRL was checked by assaying a set of samples at two time intervals 18 months apart. We found no change in BIO-PRL concentration between measurements (Campino *et al.*, 1994a). To determine the oestradiol concentration, all samples from an individual woman were analysed in duplicate in the same assay. The interassay coefficients of variation for this assay were 15.7, 14.1 and 13.5% for pools of low, medium and high concentrations respectively.

Data analysis

In previous studies we measured BIO-PRL at 0800 h, 1600 h and 2400 h in the plasma pre-suckling and also 30 min post-suckling, at 3 months post-partum (Campino *et al.*, 1994a). Oestradiol concentrations were measured in pre-suckling samples at 0800 h, 1400 h, 1800 h, 2200 h and 0200 h (Díaz *et al.*, 1991b). Since there were no significant differences in oestradiol concentrations either in pre-suckling values during the day, nor in post-suckling concentrations (data not shown), we used the average between 1400 h and 1800 h to obtain the pre-suckling oestradiol concentration at 1600 h and we used the average between 2200 h and 0200 h to obtain the pre-suckling oestradiol concentration at 2400 h. To calculate the post-suckling BIO-PRL (μg/l):oestradiol (μg/l) ratio at 0830 h, 1630 h and 0030 h, we used the BIO-PRL concentrations at these times and the average of the oestradiol concentrations calculated as described above.

The results were analysed using Wilcoxon and Friedman tests. A result was considered significant when $P < 0.05$.

Table I. Characteristics of the population and the breast-feeding pattern 3–4 months post-partum

Variable	Duration of amenorrhoea	
	>180 days (n = 5)	<180 days (n = 5)
Test day (days post-partum)	92.2 ± 18.5	100.6 ± 11.8
Mother		
age (years)	25.4 ± 1.8	24.2 ± 2.8
weight (kg)	57.2 ± 10.8	68.5 ± 7.5
height (cm)	157.0 ± 4.0	157.0 ± 8.0
Infant weight (g)		
at birth	3482 ± 271	3472 ± 242
at 90 days	6306 ± 273	6375 ± 406
Breast-feeding status		
exclusive	5	5
nursing episodes/24 h	11.2 ± 1.3	10.2 ± 0.4
suckling contacts/24 h	23.2 ± 0.8	25.2 ± 2.5
suckling duration/24 h (min)	168.8 ± 24.3	169.2 ± 59.5

Values are the mean ± SD.

Results

Characteristics of the nursing women, breast-feeding pattern and infant growth were similar in both groups (Table I).

Post-suckling prolactin concentration

The two groups of nursing women had significantly different mean values of BIO-PRL at the three periods examined ($P < 0.05$, Wilcoxon test). Post-suckling BIO-PRL at 0030 h clearly differentiated between individual nursing women who were to experience short or long amenorrhoea. The higher BIO-PRL concentration was 94 μg/l for the short amenorrhoea group and the lower BIO-PRL concentration was 133 μg/l for the long amenorrhoea group. This marked difference was attenuated when BIO-PRL was analysed at 1630 h and was not present at 0830 h (Figure 1A).

The mean value of post-suckling IR-PRL was significantly different between groups of nursing women at 1630 h and 0030 h but not at 0830 h ($P < 0.05$, Wilcoxon test) (Figure 1B). Individual concentrations of IR-PRL at these times did not differentiate between women who were to experience short or long amenorrhoea.

Oestradiol concentration

The daily mean oestradiol concentrations were significantly different between the two groups (77.1 ± 4.8 ng/l for the

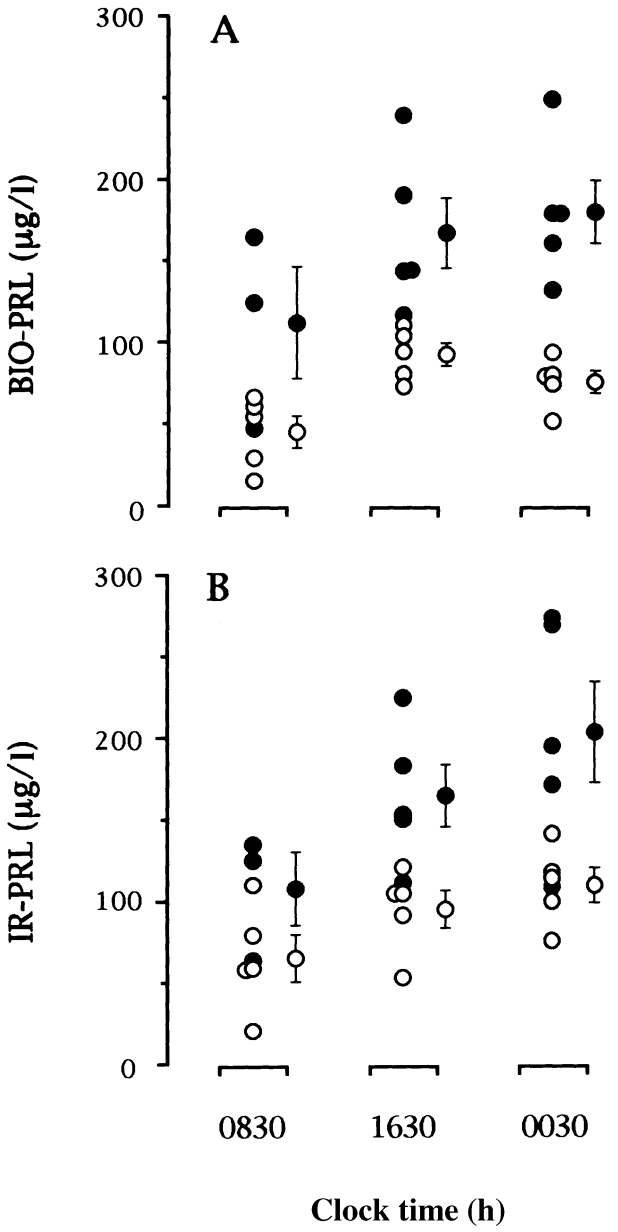

Figure 1. Post-suckling bioactive and immunoreactive prolactin (BIO- and IR-PRL) plasma concentrations (μg/l) in individual nursing women who were to experience short (<180 days, ○) or long amenorrhoea (>180 days, ●). Blood samples were drawn 30 min after the initiation of a suckling episode, at 3 months post-partum, when all women were in amenorrhoea. (**A**) BIO-PRL, individual concentrations and mean ± SE at 0830 h, 1630 h and 0030 h. (**B**) IR-PRL, individual concentrations and mean ± SE at the same times.

short amenorrhoea group versus 53.7 ± 3.2 ng/l for the long amenorrhoea group, $P < 0.05$, Wilcoxon test). Within each group, mean values were similar on the five occasions they were examined (NS, Friedman test). Nevertheless, oestradiol concentration in individual samples overlapped on four of the five occasions they were examined (Figure 2). Although there was no overlap at 1800 h the lower oestradiol concentration for the short amenorrhoea group was close to the higher oestradiol concentration for the long amenorrhoea group (62.1 and 57.0 ng/l respectively). Therefore oestradiol concentrations

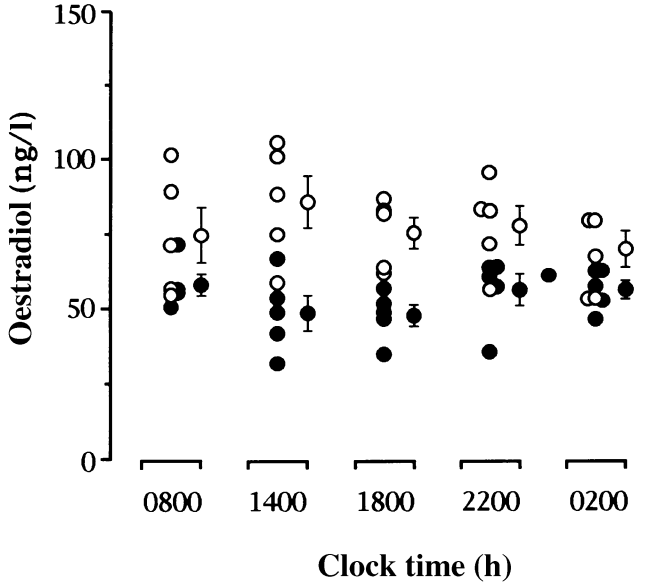

Figure 2. Plasma oestradiol concentration (ng/l) in individual nursing women who were to experience short (<180 days, ○) or long amenorrhoea (>180 days, ●), at the five time intervals indicated. Samples were drawn at 3 months post-partum, when all women were in amenorrhoea. Mean ± SE at each time are also shown.

were not useful in predicting whether an individual woman was to experience short or long amenorrhoea.

Post-suckling prolactin:oestradiol ratio

The BIO-PRL:oestradiol ratio at 0030 h and 1630 h (but not at 0830 h) clearly differentiated individual nursing women who were going to experience short or long amenorrhoea (Figure 3A). At the first two times, the ratio was always below 2000 in the short amenorrhoea group and above this threshold in the long amenorrhoea group. The greatest difference was found at 1630 h, when the highest post-suckling BIO-PRL: oestradiol ratio was 1699 for the short amenorrhoea group and the lowest ratio was 2398 for the long amenorrhoea group. At 0030 h these values were 1658 and 2145 respectively.

The IR-PRL:oestradiol ratio clearly differentiated both groups of nursing women only at 1630 h (Figure 3B). At this time, it was below 2000 in the short amenorrhoea group and above this threshold in the long amenorrhoea group. The difference between both groups was smaller than for the BIO-PRL:oestradiol ratio, since the higher post-suckling IR-PRL:oestradiol ratio was 1726 for the short amenorrhoea group and the lower ratio was 2294 for the long amenorrhoea group.

Discussion

The present study explored the possibility of utilizing post-suckling plasma concentrations of BIO-PRL, IR-PRL and basal concentrations of oestradiol measured at different times of the day to predict the duration of lactational amenorrhoea in individual fully nursing women. In the conditions of our study, the results show that in amenorrhoeic fully nursing women, at 3–4 months post-partum the post-suckling BIO-PRL, the post-suckling BIO-PRL:oestradiol ratio and the post-suckling IR-

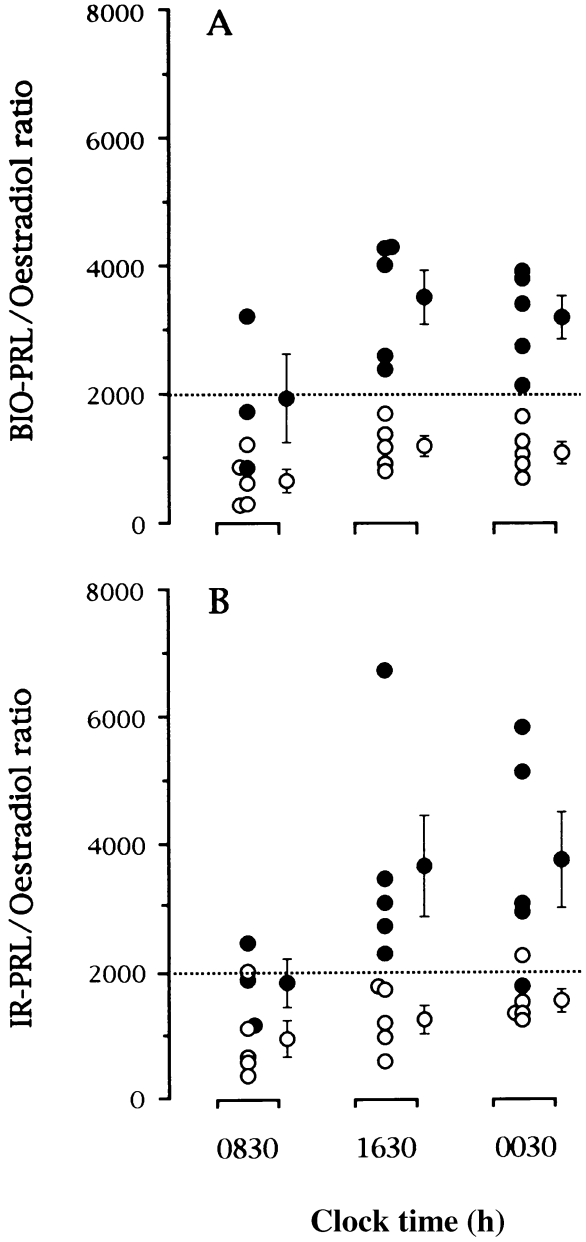

Figure 3. Post-suckling plasma bioactive prolactin (BIO-PRL) (μg/l):oestradiol (μg/l) and immunoreactive prolactin (IR-PRL) (μg/l):oestradiol (μg/l) ratios in individual nursing women who were to experience short (<180 days, ○) or long amenorrhoea (>180 days, ●). Samples were drawn 30 min after the initiation of a suckling episode, at 3 months post-partum, when all women were in amenorrhoea. (**A**) BIO-PRL:oestradiol ratio and mean ± SE at 0830 h, 1630 h and 0030 h. (**B**) IR-PRL:oestradiol ratio and mean ± SE at the same times.

PRL:oestradiol ratio can indicate which women will experience amenorrhoea lasting more than 6 months and which will recover ovarian function before 6 months post-partum.

An important factor in maintaining amenorrhoea during lactation is a high nursing frequency and absence of supplementary feedings (Bellagio Consensus Statement, 1988; Tay *et al.*, 1996). However, even if these conditions are met, some women will recover ovarian function before 6 months post-partum. Our ability to recognize them is impaired by the lack of knowledge of the mechanisms determining lactational amenorrhoea. Measurements of immunoreactive luteinizing hormone (LH) or bioactive LH concentrations (Serón-Ferré *et al.*, 1995) or immunoreactive LH pulsatility patterns in fully breast-feeding women (Díaz *et al.*, 1995) show no correlation with the duration of lactational amenorrhoea. A correlation with prolactin is found in fully breast feeding women (Díaz *et al.*, 1989; Campino *et al.*, 1994a) but not in women who give supplementary feeds (Tay *et al.*, 1996). In fully breast-feeding women the mean IR-PRL concentration secreted in response to suckling, at 3 months post-partum, was greater in the women who were to experience long amenorrhoea than in the ones who were to experience short amenorrhoea. In addition, suckling produced differences in the quality (evaluated as bioactivity) of prolactin. The discrepancy with the observations in non-fully nursing women needs to be investigated. Introduction of supplements reduces suckling duration and frequency (Howie *et al.*, 1981; Tay *et al.*, 1996). It is possible that a decrease in suckling reduces the quality of prolactin. Several prolactin isoforms are present in the plasma of nursing women (Campino *et al.*, 1994b) that may make a different contribution to total prolactin bioactivity since the immuno- and bioactivities of plasma prolactin are not equivalent (Campino *et al.*, 1994a). Alternatively, supplementary feedings may act upon some unknown factor, other than prolactin, to stimulate the recovery of ovarian function.

In the present study, we find that the correlation between prolactin and duration of lactational amenorrhoea observed during exclusive breast-feeding is detected when examining individual women. The concentration of BIO-PRL in plasma samples obtained 30 min after a suckling episode initiated at 2400 h showed a difference of 39 μg/l of BIO-PRL between the lowest concentration of the nursing women who were going to experience long amenorrhoea and the maximal concentration of the nursing women who were going to experience short amenorrhoea. At 1630 h, the minimal and maximal values, although still different, were very close. In keeping with the smaller response of IR-PRL than BIO-PRL to suckling, individual post-suckling IR-PRL *per se* at the three times examined did not differentiate individual women. Differences in the duration of lactational amenorrhoea are not explained by patient characteristics, breast-feeding patterns or rate of infant growth (Campino *et al.*, 1994a).

Daily mean oestradiol concentration was lower in the long amenorrhoea group than the short amenorrhoea group. However, there was a considerable overlap between values in individual women; thus oestradiol concentrations did not separate individual women who were going to experience short or long lactational amenorrhoea. The source of the increased oestradiol concentrations is not clear. In women who will experience short amenorrhoea, it may represent ovarian secretion or increased production by peripheral aromatization of androgens. Although the mean weight of the short amenorrhoea group was 10 kg higher than the weight of the long amenorrhoea group, the mean was not significantly different and we did not find a positive correlation between oestradiol concentration and weight. Similarly, in another study, Díaz *et al.* (1989), analysing 676 fully nursing women, did not find an association between weight and the duration of lactational amenorrhoea.

The combination of the measurements of plasma BIO- and IR-PRL and oestradiol expressed as a ratio in post-suckling samples taken at 1630 h provided two parameters that distinguished effectively between individual women according to the future duration of their amenorrhoea. A better discrimination was found when considering the post-suckling BIO-PRL:oestradiol ratio than when considering the IR-PRL:oestradiol ratio. The ratios of post-suckling BIO-PRL:oestradiol at 0030 h and 1630 h were always above 2000 in the long amenorrhoea group and below this threshold in the short amenorrhoea group. Post-suckling IR-PRL:oestradiol ratio could also distinguish the individual nursing women who will experience long or short lactational amenorrhoea at 1630 h, but not at the other times (the threshold again seems to be 2000). However, the difference between the minimal value of this ratio in the long amenorrhoea group and the maximal value in the short amenorrhoea group was smaller than that found using the post-suckling BIO-PRL:oestradiol ratio. These differences were not found in samples taken at 0830 h nor in samples taken before suckling for any of the parameters. This is in keeping with the known circadian effect of suckling on prolactin release (Díaz *et al.*, 1989b).

If the above observations are confirmed in a larger ambulatory study of fully nursing women they could provide the basis for a test. Since the measurement of IR-PRL and oestradiol are routine laboratory procedures that are even automated in some laboratories, to obtain the ratio between both measurements is not difficult.

It is important that blood samples are drawn 30 min after an episode of suckling initiated around 1600 h and lasting approximately 20 min. In addition, this episode should be separated from the previous one by at least 90 min. These time constraints provide time for prolactin to reach a baseline before suckling and take into account that prolactin response to suckling is maximal at 30 min (Díaz *et al.*, 1989b). The 90 min interval could be used to allow the women to rest after placement of a butterfly needle and may serve to prevent the response of prolactin to stress (Noel *et al.*, 1972). Operationally, blood samples should be taken at 1630 h. After separation, plasma should be stored in aliquots at –20°C. The first aliquot should be used to measure IR-PRL and oestradiol. Based upon the present results, if the post-suckling IR-PRL:oestradiol ratio is >2500 or <1500, it could predict whether the women are going to experience long or short amenorrhoea respectively. Ratios that fall between these figures could be bioassayed for prolactin, since BIO-PRL:oestradiol ratio gave a better discrimination in our study. In such cases, the second aliquot could be used to measure BIO-PRL (BIO-PRL is stable at –20°C).

The measurement of BIO-PRL has more laboratory requirements, since it uses cell culture. On the other hand, these measurements can be done by a reference centre since the length of time it takes to obtain the results is not a major constraint. The previous knowledge of the value of IR-PRL will be useful in selecting appropriate dilutions of plasma to be bioassayed, given the narrow concentration range of the standard curve of the Nb2 cell assay. The bioassay of prolactin also requires a knowledge of the concentration of human growth hormone present in the sample, to avoid overestimation of prolactin bioactivity. Although the fact that samples need to be diluted several-fold in order to perform the prolactin bioassay also decreases the interference of human growth hormone, care should be taken to avoid the known response of growth hormone to stress (Noel *et al.*, 1972).

The indicator proposed can be used to predict ovulation in fully breast-feeding, lactating women. It can be obtained in a single visit to a clinical laboratory and it requires a single blood sample, at a time that is reasonable for ambulatory clients in an urban setting. The post-suckling prolactin:oestradiol ratio would predict 1 or 2 months in advance, the recovery of fertility. In this population of women, such a knowledge permits a distinction between those nursing women who will require contraception soon, from those who will not, increasing the confidence of women to use lactational amenorrhoea as a natural contraceptive method.

To summarize, the determination of post-suckling PRL:oestradiol ratio seems to be a potential marker to predict the duration of lactational amenorrhoea in women who choose maternal milk as the sole source of nutrients for their babies.

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