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Research article

Diurnal variation in salivary progesterone in fertile Indian women

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ABSTRACT

Research question: Is there a diurnal variation in salivary progesterone levels during menstrual cycle among Indian women?
Design: A longitudinal study was carried out to measure progesterone in saliva among small cross-sectional sample (n = 31) of fertile Indian women of reproductive age comprising young adults (18–25 years, n = 11), adults (26–38 years, n = 9) and middle aged (39–45 years, n = 11). Saliva samples were collected twice daily (morning and evening) across the entire menstrual cycle of 31 women.
Results: Mean ages at enrolment and menarche were 30.6 years and 13.6 years respectively. Fifty-five percent of the women were married. The menstrual cycle range was 20–40 days. After controlling for age and menstrual cycle length, statistically significant diurnal variation in progesterone levels was observed across menstrual cycles with high levels in the morning.
Conclusions: This is the first report on salivary progesterone in subjects with Indian ethnicity and could have clinical implications for designing point of care kits for menstrual cycle management, fertility and reproduction.

1. Introduction

Saliva is a convenient specimen for monitoring the health and disease status of an individual. It contains multiple specific, soluble biomarkers making use of salivary elements as indicators of the systemic and local diseases [1,2]. Unlike blood, saliva collection is easy, non-invasive, requires no expertise and can be carried out repeatedly even by a lay person. Saliva samples are getting more consideration for testing various hormones as concentrations of hormones mostly imitate the serum unbound levels [3]. Menstrual cycle is regulated by cyclical changes in reproductive hormones secreted by the endocrine system namely the pituitary, hypothalamus and ovaries. Progesterone, secreted by the corpus luteum during the luteal phase of the menstrual cycle plays a crucial role in preparing the endometrium for implantation by decreasing myometrial contractions. Similarly, during pregnancy progesterone lowers the vascular tone of myometrium thereby preventing premature expulsion of the foetus. Skin temperature and vascular blood flow are

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Abbreviations

ELISA	Enzyme-linked immunosorbent assay
PG	Progesterone
PCOS	Polycystic ovary syndrome
PP	Polypropylene

considered to be indirect markers for different phases of menstrual cycle. It is also observed that threshold for sweating and vasodilation increases with reduction of thermal conduction and skin blood flow during luteal phase than follicular phase. These changes in the thermoregulatory set point are attributed to thermogenic properties of progesterone thus altering the body temperature [4,5]. Therefore, basal body temperature is one of the methods used by a fertile woman to plan the timing of the intercourse during the fertile window. However, studies have shown that this method is unpredictable and has a very low accuracy rate in detecting ovulation [6,7].

Prediction of menstruation, ovulation and conception in a clinical set up involves measurement of hormonal levels in the blood samples. This makes the testing invasive, cumbersome and unpopular. Researchers have thought of saliva as an alternative option for measuring hormones [8–10]. It has also been used to assess the Corpus luteum function during the mid-luteal phase [11]. Few researchers have also conducted studies on the fluctuating nature of progesterone (PG) secretion during daytime periods [12,13]. Diurnal variation in PG levels at different phases and timings of a menstrual cycle has clinical implications in the field of infertility treatment, ovulation and menstruation.

For the measurement of salivary hormones several analytical techniques have been reported. Many studies in the past have measured reproductive hormones using radioimmunoassay [14,15]. However, this assay is time consuming and requires the use of radioactive material. Chemiluminescence [16] and ELISA assays [8,17] have largely replaced radioimmunoassay. Liquid chromatography tandem mass spectrometry (MS) assays have been developed for the simultaneous estimation of salivary steroidal hormones [18,19]. Despite lower specificity and some cross-reactivity, immunoassays [20] are still preferred over MS due to their technical ease, lower cost of reagents and high throughput.

During the literature search it was realised that there are few studies comparing salivary PG among different ethnicities [21–23] and there is scarcity of data on salivary PG levels in the Indian women [24,25]. Our research group is primarily working towards finding diagnostic applications of progesterone for menstrual and reproductive health. Since saliva is a heterogeneous sample, it is necessary to standardise the time of sample collection. Hence, we designed this study to understand the variability in the length of menstrual cycle and diurnal variation in progesterone levels. Study subjects were classified in three distinct reproductive age groups: young adults (age 18–25 years), adults (26–38 years) and middle aged (39–45 years).

2. Materials and methods

2.1. Subjects

The study was carried out among the female staff members (faculty members, teachers and nurses) and students staying in the campus of the BKL Walawalkar Hospital and Rural Medical College situated in the Konkan region of western Indian state of Maharashtra. Thirty-one women volunteered to participate in this study. The inclusion criteria were absence of reproductive problems such as polycystic ovary syndrome (PCOS), absence of dental or oral diseases including mouth ulcers, absence of drug and tobacco usage. Pregnant women and those using contraceptive pills were also excluded from the study. A group meeting was held before the commencement of the study and participants were fully informed about the objectives of the study and discomforts associated during the sample collection procedure. Each participant provided a written informed consent which was approved by the institutional ethical committee of the hospital.

Each woman was asked to provide the upcoming expected menstrual period date. List of expected menstrual period dates was generated and women were called to the research centre daily to provide saliva samples (fasting/non fasting state) beginning the 1st day of the menstrual cycle. Each woman was expected to provide 2 saliva samples daily (morning and evening). Daily sample collection continued till the 1st day of the next menstrual cycle. Expected number of samples to be collected from 31 women of varying menstrual cycle lengths was 877 each in the morning and evening. We collected 607 samples in the morning and 660 samples in the evening, giving us the sample collection rate of 69.2% and 75.2% respectively; 548 paired samples (both morning and evening) were obtained with a paired sample collection rate of 62.5%. Sample collection time in the morning varied from 6 a.m. to 10 a.m., and 93% of the samples were collected between 8 a.m. and 10 a.m. Sample collection time in the evening varied from 3 p.m. to 7 p.m., and 75.2% of the samples were collected between 4 p.m. and 6 p.m. We enrolled 31 women comprising young adults (18–25 years, $n = 11$), adults (26–38 years, $n = 9$) and middle aged (39–45 years, $n = 11$).

2.2. Saliva sample collection

Participants were asked to rinse their mouth with water 5 min prior to the sample collection. They were asked to avoid use of mouthwashes, lipsticks and consumption of food, drinks and chewing gums at least 30 min before submitting saliva samples. Participants provided 2–3 ml of unstimulated drool saliva sample in a wide mouthed polypropylene (PP) container. Each saliva sample

was transferred in a 5.0 ml PP centrifuge tube (Tarsons Products, India) and centrifuged at 800×g for 5 min at 4 °C. Aliquots were prepared in 1.8 ml PP cryovials (Tarsons Products, India) and stored at −20 °C until analysis.

2.3. Laboratory measurements of PG

On the day of analysis, saliva samples were thawed and centrifuged at 3381×g for 10 min at 4 °C. Salivary progesterone concentration expressed in pg/ml was determined by a commercially available salivary progesterone enzyme immunoassay kit (SLV-2931 by DRG, Germany). The intra and inter batch coefficients of variation (CV) were 19.7% and 21.9% respectively. These CVs were calculated on low control samples provided in the ELISA kit by the manufacturer.

2.4. Ethics

Institutional Ethic Committee of BKL Walawalkar Hospital & Rural Medical College granted the permission to carry out the study. IEC is registered with the Government of India. The registration code is EC/755/INST/MH/2015/RR-18.

2.5. Statistical analysis

We have represented data on basic characteristics of women by mean, standard deviation and percentage. Distribution of salivary PG was skewed. It has been argued that the proportional changes in progesterone hormone affect the outcomes and log transformation linearizes the hormonal effects [26,27]. Outcome in our study was diurnal variation. We normalised salivary PG using log transformation LOG_{10} (salivary PG + 2). Difference in log transformed concentrations of morning and evening salivary PG reflected the additive effect of log transformed salivary PG levels. We also quantified the diurnal variation by % difference of morning concentrations of salivary PG from evening concentrations (pg/ml) of salivary PG by calculating {(morning concentration-evening concentration)/(evening concentration)}x100. We analysed the data using 2 methods. In the first method we identified luteal and follicular phases of the menstrual cycle. The luteal phase of the analysed menstrual cycle was assumed to begin 14 days before the first day of the next menstrual cycle [21]. Period before luteal phase i.e., follicular phase was standardised using tertiles with each tertile representing 1/3rd period of the follicular phase. We were not able to identify the ovulatory period as we did not have ultrasound or luteinizing hormone (LH) measurement data. In the second method, we controlled for age and menstrual cycle length by creating cycle length and age group specific deciles (D_1 - D_{10}). Each decile represented 1/10th or 10% period of the menstrual cycle length. Diurnal variation in PG (difference between evening and morning levels) across the menstrual cycle was analysed for each tertile/decile and luteal phase using repeated measurement analysis. P value of <0.05 was considered as statistically significant. Effect size for the paired comparison between salivary PG concentrations in the morning and evening was estimated using Cohen's d and was calculated only for the statistically significant paired comparisons. They were interpreted using the classification suggested by Ref. [28]. Data analysis was carried out using SPSS 25.0 (IBM, Chicago) and STATA 13.0 (STATA corporation, Texas).

3. Results

Thirty-one women of reproductive age (18–45 years) were enrolled in the study. Mean age at enrolment was 30.6 year and mean age at menarche was 13.6 years. Mean height, weight and body mass index were 152.5 cm, 50.4 kg and 21.7 kg/m^2 respectively (Table 1). Fig. 1 shows daily morning as well as evening salivary PG levels of 3 individual women of different age but same menstrual cycle length of 27 days. It indicates diurnal variation in each age group. Table 2, shows a summary of salivary PG levels in the morning and evening as well as diurnal variation within the entire study sample as well as per each age group according to follicular and luteal phases of the menstrual cycle. Statistically significant diurnal variation was observed in the entire group of women and group of young adult women for both follicular as well as luteal phase. Adults and middle-aged groups showed significant diurnal variation only in the

Table 1
Basic characteristics of women enrolled (n = 31).

Age (years)	30.6 (8.4)
Age categories	
18–25 years	11 (35.5%)
25–38 years	9 (29.0%)
38–45 years	11 (35.5%)
Age at menarche (years)	13.6 (1.6)
Height (cm)	152.5 (8.4)
Weight (kg)	50.4 (9.7)
BMI (kg/m^2)	21.7 (4.0)
Marital status	
Married	17 (54.8%)
Unmarried	14 (45.2%)

Mean (SD) for continuous variables; n (%) for categorical variables; SD: standard deviation.

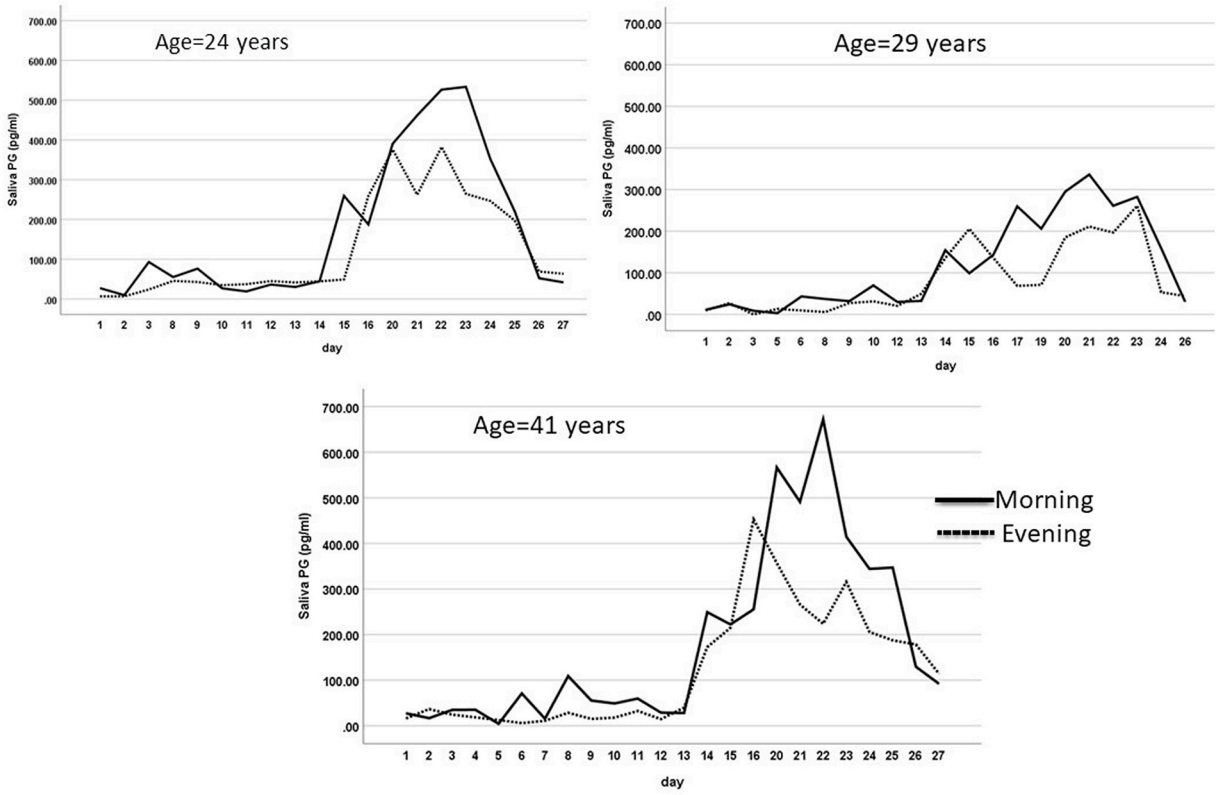

Fig. 1. Salivary PG (pg/ml) across menstrual cycle of 3 individual women from 3 distinct age groups but same menstrual cycle length cycle length of 27 days
Legend: Bold curve shows salivary PG concentrations in the morning and dotted curve shows salivary PG concentrations in the evening across menstrual cycle.

luteal phase. The effect size varied from 0.20 to 0.47 which are interpreted as small to medium [28]. Same data has been shown in Fig. 2A, 2B, 2C respectively for women in each age group with mean salivary PG levels with 95% confidence intervals across the menstrual cycle. Statistically significant diurnal variation was observed on days −8, −6 and −4 of the luteal period for women in younger age group. Diurnal variation with confidence probability of diurnal difference between 90 and 95% was also observed on days 7, 8, 9, 10, 11 and 12 of luteal period in the adult age group. No statistically significant diurnal variation was observed in the middle age group. Table 3, shows a summary of salivary PG levels in the morning and evening as well as diurnal variation of each individual woman according to each menstrual cycle length group, according to follicular and luteal phases of the menstrual cycle. We were able to observe large effect sizes (0.5 or above) at individual levels. We also created menstrual cycle length and age group specific deciles and analysed diurnal variation in salivary PG across deciles (Figure-3). The actual statistical significance with p value of at least 0.05 was observed in all the deciles. We have also summarised the decile data in Table-4.

Out of 548 paired samples, 373 (68%) paired samples showed diurnal variation with higher mean salivary PG levels in the morning; 240.5% in the follicular phase and 134% in the luteal phase (Data not shown). Out of 297 paired samples from luteal phase showing diurnal variation, 199 (67%) exhibited higher salivary PG levels in the morning (Data not shown).

4. Discussion

We have shown diurnal variation in salivary PG levels in fertile Indian women from 3 distinct age groups. The results indicated more diurnal variation in the second approach of menstrual cycle deciles (Fig. 3) than those obtained by identifying the luteal phase (Fig. 2A, 2B, 2C). Thus, differences in statistical conclusions of 2 methods could be due to the number of data points used while calculating tertiles or deciles. We did not consider PG levels at night time as we did not enrol women working in the night shifts. However, few studies have shown adverse effects of shift work in few fertile women leading to irregular and abnormally long menstrual periods [29]. Progesterone has lot of clinical implications in regulation of menstrual cycle, release of oocyte and facilitation of implantation, augmenting uterine growth and reduction in myometrial contractility, preparedness for conception and maintaining pregnancy, breast development for milk secretion and modulation in bone mass thus endorsing its role in female growth and reproduction [30]. Female reproductive hormones display an endogenous circadian regulation and these diurnal fluctuations are usually not considered while concluding the results. Diurnal rhythmicity of progesterone and oestrogen is more during the follicular phase of the

Table 2
Morning and evening Salivary PG (pg/ml) concentrations, diurnal variation and effect size across follicular and luteal phase according to age groups (n = 31).

Age group	Number of subjects	Number of paired samples	Follicular phase						Luteal phase*					
			Morning	Evening	Diurnal variation		P	Effect size	Morning	Evening	Diurnal variation		P	Effect size
					Difference between morning and evening	% difference from evening					Mean	Mean % difference from evening		
All	31	548	32.5 (43.9)	22.9 (31.4)	9.6 (47.7)	41.9	0.001	0.20	185.9 (168.1)	142.3 (128.3)	43.6 (111.4)	30.64	0.000	0.39
18–25	11	205	47.5 (62.6)	23.1 (23.2)	24.3 (61.0)	105.1	0.000	0.39	171.5 (153.8)	121.4 (114.3)	50.1 (108.8)	41.27	0.000	0.46
25–38	9	164	22.3 (14.8)	18.2 (15.6)	4.1 (18.66)	22.5	NS	–	224.4 (144.7)	175.4 (125.8)	49.0 (103.1)	27.94	0.000	0.47
38–45	11	179	22.9 (22.6)	27.8 (49.3)	−4.9 ()	−17.9	NS	–	169.3 (193.4)	136.3 (138.7)	33.0 (127.9)	24.21	0.001	0.25

Mean (Standard deviation) for morning, evening and diurnal difference data.
*: Luteal phase was defined as per [21].
p: p value for difference between morning and evening salivary PG.
NS: Not significant.
-: Effect size was not calculated as p was not significant.

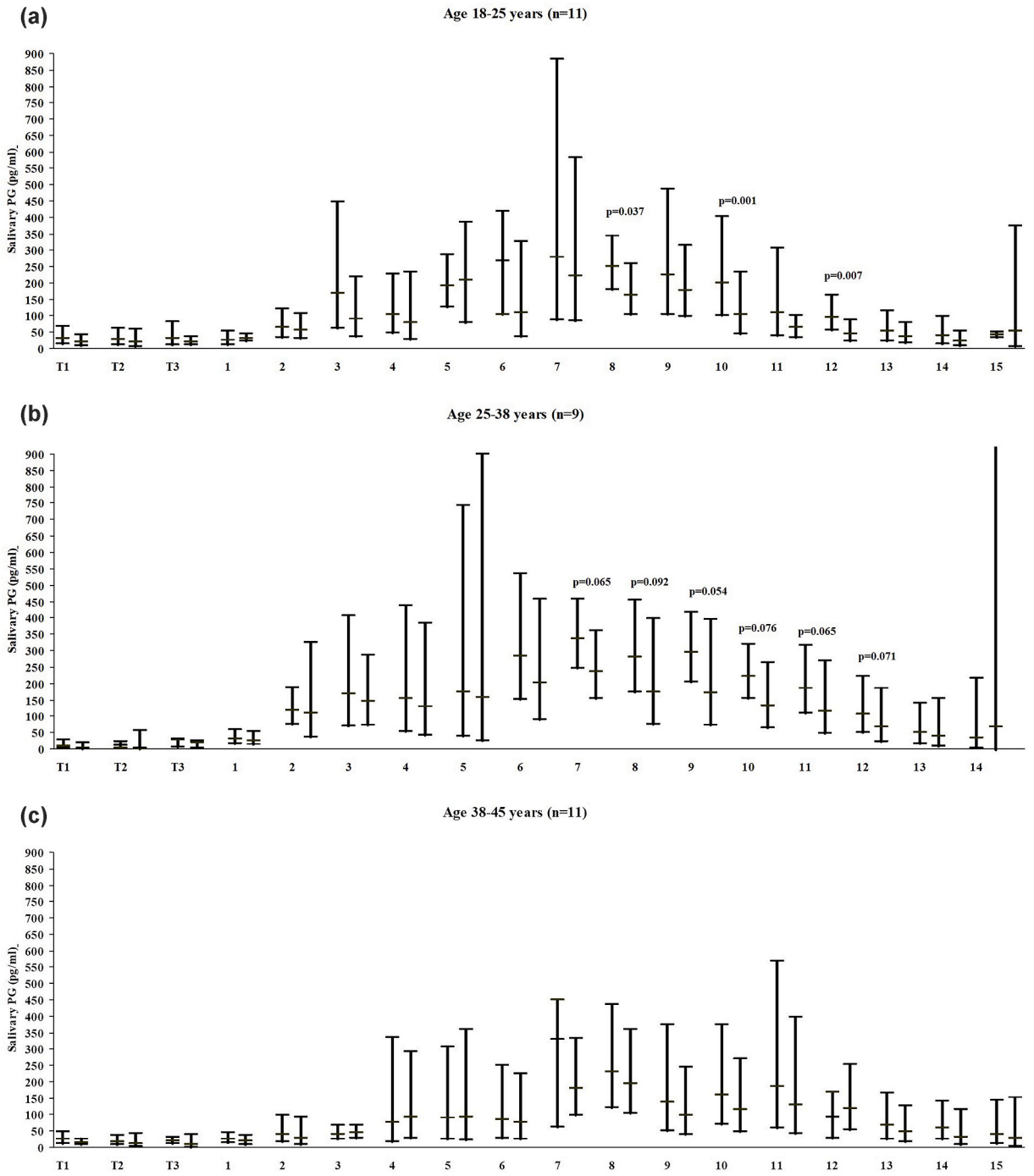

Fig. 2. 2A Diurnal variation in salivary PG (pg/ml) across menstrual cycle (age group 18–25 years). 2B Diurnal variation in salivary PG (pg/ml) across menstrual cycle (age group 25–38 years). 2C Diurnal variation in salivary PG (pg/ml) across menstrual cycle (age group 38–45 years) Legend:T1, T2, T3: 1st, 2nd, 3rd tertile of follicular phase respectively. 1, 2, 3, …, 14 are days of luteal phase. p values are for the difference between morning and evening salivary PG levels.

menstrual cycle than in the luteal phase [29]. However, mid luteal PG measurements also showed a pulsatile pattern [13]. Studies have shown diurnal variation in serum PG levels in fertile women. A study on women on IVF treatment showed fluctuation in serum PG levels from 7 nmol/l to as high as 128 nmol/l within a short time [12]. As per our knowledge there is less data on the diurnal variation of progesterone in saliva [31,32]. Ours is probably the first report among non-pregnant Indian women. Elevated levels of progesterone are known to emerge in the morning and those of oestrogen at night time in serum [12]. In our study, elevated salivary PG levels were

Table 3
Summary of morning and evening salivary PG (pg/ml) and diurnal variation across follicular and luteal phase according to individual subjects and all the subjects within each menstrual cycle length group (n = 31).

Cycle length	Number of subjects within menstrual cycle length group	Paired samples (n)	Follicular phase						Luteal phase*					
			Morning	Evening	Diurnal variation		P	Effect size	Morning	Evening	Diurnal variation		P	Effect size
					Difference between morning and evening	% difference from evening					Difference between morning and evening	% difference from evening		
20	All	6	4.3	9.8	−5.4	−55.1	NA	NA	103.8 (50.2)	105.3 (43. 6)	−1.6 (71.4)	−1.5	NS	–
23	Subject-1	20	18.7 (9.7)	10.7 (5.2)	7.9 (12.6)	73.8	NS	–	133.1 (133.1)	81.4 (82.1)	51.7 (143.5)	63.5	NS	–
23	Subject = 2	11	27.8 (12.6)	26.9 (10.2)	0.8 (2.5)	2.9	NS	–	96.5 (112.6)	116.0 (132.7)	−19.4 (94.1)	−16.7	NS	–
23	All (n = 2)	31	20.5 (10.3)	13.9 (8.9)	6.5 (11.5)	46.8	NS	–	116.7 (122.6)	97.0 (106. 2)	19.7 (126.2)	20.3	NS	–
24	Subject-1	15	8.2 (3.5)	3.7 (1.6)	4.6 (4.4)	124.3	NS	–	112.9 (69.2)	43.6 (15.9)	69.3 (59.9)	158.9	0.008	1.15
24	Subject-2	15	168.0 (112.3)	44.8 (23.0)	124.0 (121.5)	276.8	0.036	1.02	451.4 (209.3)	210.9 (139.8)	240.4 (158.1)	114.0	0.004	1.52
24	All (n = 2)	30	101.9 (117.2)	27.6 (27.2)	74.2 (108.8)	268.8	0.038	0.68	272.2 (227.8)	122.3 (126.9)	149.9 (143.1)	122.6	0.001	1.04
25	Subject-1	19	15.4 (7.1)	15.5 (10.1)	−0.1 (14.0)	−0.6	NS	–	255.6 (122.9)	164.2 (68.9)	91.2 (117.6)	55.5	0.048	0.77
25	Subject-2	21	8.2 (7.3)	4.3 (4.8)	3.9 (2.7)	90.7	0.009	1.4	73.4 (48.7)	72.3 (55.9)	1.1 (34.0)	1.50	NS	–
25	Subject-3	18	14.1 (5.9)	6.9 (5.6)	7.2 (5.1)	104.3	0.005	1.4	221.1 (143.9)	130.4 (106.1)	90.7 (59.8)	69.6	0.002	1.51
25	All (n = 3)	58	12.9 (7.2)	9.4 (8.7)	3.5 (9.4)	37.2	NS	–	169.2 (132.4)	115.9 (84.3)	53.3 (84.5)	46.0	0.001	0.63
26	Subject-1	21	14.3 (5.1)	15.7 (6.3)	−1.4 (9.6)	−8.9	NS	–	130.3 (67.7)	129.2 (122.6)	1.0 (85.6)	0.8	NS	–
26	Subject-2	8	21.4	24.9	−3.4	−13.7	NA	NA	100.4 (58.6)	68.9 (42.0)	31.5 (28.7)	45.7	0.027	1.09
26	Subject-3	19	44.2 (33.9)	125.8 (76.4)	−81.5 (71.5)	−64.8	0.006	−1.13	598.6 (222.1)	455.7 (79.3)	142.9 (193.9)	31.4	NS	–
26	All (n = 3)	48	30.5 (28.5)	74.2 (77.9)	−43.7 (65.3)	−58.9	0.009	−0.6	261.4 (256.7)	210.4 (188.2)	50.9 (131.4)	24.2	NS	–
27	Subject-1	4	3.0	13.2	−10.1	−76.5	NA	NA	234.2 (65.9)	170.4 (106.4)	63.8 (42.1)	37.4	NS	–
27	Subject-2	18	46.3 (29.8)	29.2 (7.2)	17.0 (27.4)	58.2	NS	–	288.2 (195.1)	195.4 (132.0)	92.8 (107.3)	47.5	0.032	0.86
27	Subject-3	19	43.3 (34.8)	21.3 (11.0)	22.0 (34.7)	103.3	NS	–	356.9 (185.1)	247.2 (101.7)	108.9 (176.1)	44.1	NS	–
27	All (n = 3)	41	42.1 (32.1)	24.2 (4.1)	17.8 (30.4)	73.6	0.033	0.58	310.7 (178.8)	214.7 (115.4)	96.0 (133.3)	44.7	0.002	0.72
28	Subject-1	8	31.1 (12.7)	23.4 (17.8)	7.7 (5.2)	32.9	NS	–	45.9 (5.1)	19.2 (5.4)	26.7 (4.2)	139.1	0.000	6.20
28	Subject-2	18	21.7 (7.2)	11.9 (10.2	9.7 (10.2)	81.5	0.021	0.95	180.4 (91.1)	196.1 (92.8)	−15.7 (123.7)	−8.0	NS	–

(*continued on next page*)

Table 3 (*continued*)

Cycle length	Number of subjects within menstrual cycle length group	Paired samples (n)	Follicular phase						Luteal phase*					
			Morning	Evening	Diurnal variation		P	Effect size	Morning	Evening	Diurnal variation		P	Effect size
					Difference between morning and evening	% difference from evening					Difference between morning and evening	% difference from evening		
28	All (n = 2)	26	23.4 (8.5)	14.1 (11.7)	9.4 (9.2)	66.7	0.007	1.02	128.7 (97.4)	128.1 (114.2)	0.60 (96.9)	0.5	NS	–
29	Subject-1	20	13.9 (6.5)	6.6 (2.7)	7.3 (5.8)	110.6	0.001	1.25	160.0 (131.7)	141.6 (163.9)	18.4 (148.3)	13.0	NS	–
29	Subject-2	26	6.8 (3.1)	4.2 (2.3)	2.6 (2.3)	61.9	0.004	1.13	50.4 (24.5)	53.9 (55.3)	−3.6 (54.2)	−6.7	NS	–
29	Subject-3	23	17.9 (12.9)	11.9 (6.0)	6.0 (14.1)	50.4	NS	–	169.3 (180.5)	120.4 (125.9)	48.9 (203.8)	40.6	NS	–
29	Subject-4	19	22.1 (10.0)	14.7 (6.4)	7.4 (13.9)	50.3	NS	–	152.2 (106.5)	86.0 (68.8)	66.1 (60.9)	76.6	0.018	1.08
29	All (n = 4)	88	15.0 (10.3)	9.2 (6.1)	5.8 (10.1)	63.0	0.000	0.57	122.4 (127.4)	94.5 (107.7)	27.9 (126.3)	29.5	NS	–
30	Subject-1	23	20.9 (13.1)	11.1 (6.4)	9.9 (10.3)	89.2	0.015	0.96	124.9 (105.7)	90.2 (69.6)	34.8 (62.6)	38.6	NS	–
30	Subject-2	16	35.3 (9.96)	39.0 (28.0)	−3.7 (27.9)	−9.5	NS	–	87.8 (71.5)	55.3 (37.50	32.5 (48.9)	58.8	NS	–
30	Subject-3	21	112.1 (93.1)	35.3 (45.9)	76.8 (109.1)	217.6	0.033	0.70	61.9 (73.9)	28.6 (8.5)	33.4 (72.9)	116.8	NS	–
30	All (n = 3)	60	59.6 (69.9)	28.9 (33.7)	30.7 (70.3)	106.2	0.03	0.43	97.4 (91.6)	63.5 (57.0)	33.9 (61.2)	53.4	0.011	0.55
31	Subject-1	20	33.9 (7.6)	16.7 (7.2)	17.2 (7.6)	103.0	0.000	2.26	205.5 (111.9)	201.0 (95.3)	4.5 (77.8)	2.2	NS	–
31	Subject-2	22	25.8 (12.2)	33.5 (17.1)	−7.7 (16.7)	−23.0	NS	–	202.5 (105.9)	217.1 (115.70	−14.6 (104.3)	−6.7	NS	–
31	All (n = 2)	42	29.5 (10.9)	25.8 (15.8)	3.7 (18.2)	14.3	NS	–	203.9 (105.8)	209.1 (103.2)	−5.1 (89.8)	−2.4	NS	–
32	Subject-1	23	14.8 (5.7)	13.2 (3.7)	1.6 (5.1)	12.1	NS	–	102.3 (69.8)	79.3 (81.3)	22.9 (74.8)	28.9	Ns	–
32	Subject-2	16	37.3 (14.5)	18.9 (5.4)	18.4 (15.6)	97.4	0.012	1.17	197.7 (93.5)	210.0 (118.2)	−12.3 (51.1)	−5.9	NS	–
32	Subject-3	22	18.4 (13.3)	22.0 (15.6)	−3.6 (19.6)	−16.4	NS	–	333.2 (168.1)	324.1 (123.3)	9.2 (143.2)	2.8	NS	–
32	All (n = 3)	61	22.7 (14.7)	17.8 (10.1)	4.9 (16.7)	27.5	NS	–	212.7 (155.4)	203.8 (150.0)	8.9 (100.2)	4.4	NS	–
33	All (n = 1)	20	37.8 (24.2)	28.4 (21.3)	9.3 (37.9)	32.7	NS	–	331.6 (173.4)	230.7 (108.7)	100.9 (127.1)	43.7	0.044	0.79
36	All (n = 1)	13	61.5 (33.3)	36.7 (14.8)	24.8 (31.3)	67.6	NS	–	132.8 (94.5)	83.3 (30.9)	49.5 (72.9)	55.4	NS	–
40	All (n = 1)	24	16.5 (6.5)	8.5 (4.3)	8.0 (3.4)	94.1	0.000	2.28	106.4 (71.0)	57.0 (34.6)	49.4 (60.9)	84.7	0.031	0.81

Mean (Standard deviation) for morning, evening and diurnal difference data.
*: Luteal phase was defined as per [21].
p: p value for difference between morning and evening salivary PG.
NS: Not significant.
-: Effect size was not calculated as p was not significant.

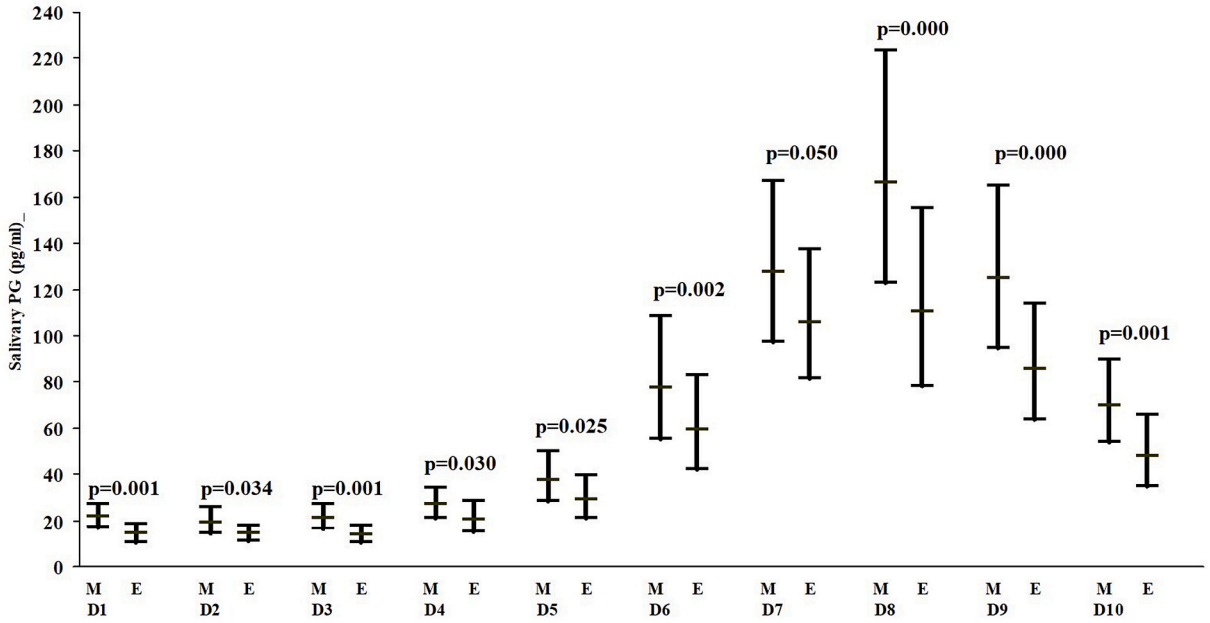

Fig. 3. Diurnal variation across menstrual cycle length and age group specific deciles (n = 31)
Fig. 3 Legend:
M: Morning; E: Evening; D1, … …., D10 are menstrual cycle length and are group specific deciles.
p values are for the difference between morning and evening salivary PG levels.

observed in the morning however statistical significance was observed only at selected time points. These observations may guide us in deciding the timing of sample collection for hormone measurements for accurate results and its clinical interpretations.

4.1. Strengths and limitations

Longitudinal study and dense within subject sampling was a major strength. Many studies on diurnal variation have relied on samples provided by subjects as per convenience. Our saliva sample collection was performed in the laboratory in the presence of trained technical staff thus ensuring adherence to sample collection protocol. All samples were processed and stored immediately. However, sample size was small for an epidemiological study. We were unable to accurately estimate the menstrual cycle phases using valid techniques like ultrasound. We could not ensure fasting status at the time of saliva sample collection because of the shift work pattern of subjects. It was a single cycle study hence we were unable to analyse seasonal variations. We did not use any normalisation factors (salivary flow rate, total protein or saliva osmolality) during salivary PG analysis. Previous studies [20] have reported low accuracy in immunoassays for measuring low concentrations of steroids. We have also observed high inter assay CV for low control samples.

Table 4
Summary of morning and evening salivary PG (pg/ml) across menstrual cycle length and age group specific deciles (n = 31).

			Diurnal variation	
Decile number	Morning	Evening	Mean	Mean % difference from evening
1	32.2 (41.6)	22.2 (27.8)	9.9	145.9
2	35.1 (61.3)	20.2 (26.5)	14.8	111.8
3	33.2 (46.8)	23.2 (38.2)	9.9	118.9
4	40.9 (55.3)	38.1 (54.3)	2.9	120.7
5	71.5 (105.2)	57.0 (77.4)	14.5	211.7
6	148.4 (174.8)	113.6 (132.8)	34.7	58.2
7	188.6 (152.6)	152.8 (124.5)	35.8	50.2
8	246.6 (185.5)	182.2 (141.6)	64.4	79.6
9	201.8 (192.9)	142.3 (137.7)	59.4	70.3
10	102.6 (87.9)	82.3 (88.8)	19.8	147.0

Mean (Standard deviation) for morning and evening data.
Mean for diurnal variation data.
* Luteal phase was defined as defined in Ref. [21].

5. Conclusions

In conclusion, our results indicate that saliva can be a clinically useful specimen for measuring reproductive hormones. Our data could be used to develop reference ranges for salivary PG over the entire menstrual cycle in women of reproductive age. Sixty seven percent of the paired samples in our study exhibited higher salivary PG levels in the morning. Previous studies [11] have suggested cut-off values for salivary PG for the luteal phase insufficiency. In our data we have reported higher luteal phase salivary PG in the morning samples though effect size was small. This difference might be crucial in determining salivary PG levels in individuals with suboptimal ovarian function. Hence, we would recommend salivary sample collection in the morning. Study results would be useful for therapeutic and diagnostic applications in menstruation and reproduction.

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Declaration of competing interest

None of the authors have any competing interests to declare.

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