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Daily levels of sex hormones in 15 subfertile women formulate a menstrual cycle profile predominant with progesterone secretion

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Abstract

Introduction: Changes in sex hormone secretions during the menstrual cycle may affect fertility. It has been shown that a prematurely raised progesterone (P4) level after therapeutic injection of human chorionic gonadotropin caused changes in endometrial gene expression and lowered the pregnancy rate. The aim of the present study was to investigate the complete menstrual patterns of P4 together with its derivatives testosterone (T) and oestradiol (E2) in subfertile women during their natural cycles.

Material and methods: Daily serum levels of P4 (ng/mL), T (ng/mL), E2 (pg/mL), and sex hormone binding protein (SHBG, nmol/L) were measured throughout a single 23–28-day menstrual cycle in 15 subfertile women aged 28–40 years with patent oviducts and normospermic partners. Knowing SHBG levels, the free androgen (FAI) and free oestrogen (FEI) indexes were calculated for each cycle day in each patient.

Results: Baseline (cycle day one) levels of luteinising hormone (LH), thyroid stimulating hormone (TSH), P4, and T were comparable with reference intervals for a normal cycle, whereas follicle stimulating hormone (FSH), E2, and SHBG exceeded those. During cycles, the levels of P4 correlated positively with E2 levels ($r = 0.38$, $p < 0.05$, $n = 392$) and negatively with T ($r = –0.13$, $p < 0.05$, $n = 391$). T correlated negatively with E2 ($r = –0.19$, $p < 0.05$, $n = 391$). Menstrual cycle phases were hidden. The curve of the mean/median daily levels of P4 rose prematurely, was parallel with the E2 rise, and culminated closely, but with more than 4 times greater amplitude of P4 (2571% of baseline levels in day 16) than of E2 (580% in day 14). In turn, the curve of T declined in a U-shaped manner with a nadir (–27%) on day 16. Averaged daily levels of FEI, but not FAI, varied significantly between 23 and 26 days long and the 27–28-day cycles.

Conclusions:

1. Throughout the entire menstrual cycle length in subfertile women, P4 secretion predominates quantitatively over secretions of the remaining sex hormones when menstrual cycle phases are hidden.
2. The rise of E2 secretion is in parallel with the P4 rise, but with 4 times lower amplitude of E2.
3. T secretion declines and is inversely related to both P4 and E2 secretions.
4. Changes in E2 bioavailability are related to menstrual cycle length. **(Endokrynol Pol 2023; 74 (1): 106–112)**

Key words: *progesterone; testosterone; oestradiol; subfertility*

Introduction

Subfertility is the inability to conceive after one year of unprotected intercourse, with 25% of cases not explained by oviduct inadequacy, anovulation, or low sperm count [1]. Quantitative changes in ovarian steroid hormone secretions may affect menstrual cycle fecundity. It has been shown that a premature rise of progesterone (P4) secretion in a day of cycle stimulation with human chorionic gonadotropin in the in vitro fertilization procedure (IVF) was associated with lowered pregnancy and implantation rates [2]. Alterations of endometrial gene expressions, important for endometrial receptivity, have been postulated to be a direct cause [3].

In nature, P4 is a hormone precursor for testosterone (T) and E2 biosynthesis in the gonadal steroidogenic pathway [4–6]. These hormones formulate separate menstrual cycle-related secretion patterns but are rarely investigated simultaneously. The objective of the current study is to gain an understanding of the complete pattern of P4 secretion in relation to the patterns of T and E2 during natural cycles in subfertile women. The menstrual pattern of the circulating

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sex hormone binding globulin (SHBG), a protein that binds but also inactivates T and E2, was investigated simultaneously.

Material and methods

This is a prospective, observational, single-cycle study in a clinical sample of women with regular cycles, patent oviducts, and partnered with a man having normal sperm count, yet experiencing no pregnancy after a year of unprotected intercourse. All the participants had proven normal oviduct patency before enrolment.

Patient enrolment

Twenty-one healthy participants were approached, all with university education. Six dropped out because of the burden. Fifteen women were enrolled. They denied sleep problems, hot flushes, or night sweats. They accepted daily venous blood sampling throughout one entire menstrual cycle by giving their signed informed consent. In return, they were granted, free of charge, medical consultations and a complete hormone analysis panel prior to and during a potential infertility treatment. All revealed normal blood morphology before enrolment and after the study was complete.

None had used hormonal contraception for the previous 2 years. All were non-smokers and denied excessive alcohol consumption. Exclusionary criteria included athleticism, vegetarian diet, regular use of medication including aspirin, and a history of chronic disorders, including endocrine gynaecological diseases. Body mass index (BMI) was calculated according to the formula kg/m^2 where kg is a person's weight in kilograms and m^2 is their height in metres squared.

Semen of the partners of all participants was obtained by masturbation. It was performed twice with a 3-week interval, all after 4 days of ejaculatory abstinence. Analysis was performed according to WHO-advised methods and criteria.

The study project and its procedures were approved by the Ethics Committee of the Medical University of Lodz. All procedures performed in the study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments. This paper does not contain study with animals.

Hormone measurements

The first day of menstrual bleeding was taken as day 1 of the cycle, and the cycle length as the number of days from day 1 to the day preceding the next menstruation. Venous blood was taken daily throughout the entire menstrual cycle before 10:00 a.m. in the spring season (April–June).

The serum was separated immediately after phlebotomy and stored at –80°C until a single-run assay of each of the hormones. All assays were performed in duplicate.

Serum concentrations of follicle stimulating hormone (FSH), luteinising hormone (LH), and thyroid stimulating hormone (TSH) were measured once at the cycle baseline (day 1) by chemiluminescence immunoassay with a modular E170 automated analyser (Roche Diagnostics, Mannheim, Germany).

Serum P4, T, and E2 were measured by an Immunolite 2000 analyser (Siemens Healthcare Diagnostics Ltd, Frimley, United Kingdom). The P4 measurement sensitivity ranged between 0.05 and 60 ng/mL with 1.9% of intra-assay variability (Cobas) and specificity that included cross-reactivity with 0% of androstenedione, 0% of E2, and 0.075% of T.

The serum T measurement sensitivity was between 0.07 and 15 ng/mL with 2.7% intra-assay variability (ADIVA), and cross-reactivity that included 1.39% of androstenedione, 0.26% of E2, and 0.01% of P4.

The serum E2 measurement sensitivity was between 5 and 3000 pg/mL with 3.5% of intra-assay variability (Cobas), and cross-reactivity that included 0.005% of androstenedione, 0% of T, and 0.004 % of P4.

Serum concentrations of SHBG were measured by an Immunolite 2000 analyser (Siemens Healthcare Diagnostics Ltd, Frimley, United Kingdom). The serum SHBG measurement sensitivity was between 1.6 and 180 nmol/L.

Free oestrogen index (FEI) and free androgen index (FAI) were calculated by dividing the total concentration of the hormone (nmol/L) by concentration of SHBG (nmol/L) and multiplying by 100.

One cycle of each woman was investigated. Each cycle lasted between 23 and 28 days. Until day 23 each day consisted of 15 participants. Following day 23, the number of participants per day was lower: days 24 and 25 were represented by 13 participants, days 26 and 27 by 9 participants, and day 28 by 4 participants. No intermittent gaps were present.

To assure synchronization of the cycles, a forward day method of cycle alignment was applied for plotting daily hormone concentrations against each subsequent cycle day, beginning from day 1.

Statistical analysis

In total, 1662 data points were collected. The results were analysed as median and ranges, and expressed as the mean ± standard error of the mean (SEM) in graphs. Comparisons within the cycle involved analysis of variance (ANOVA). Associations between hormone concentrations were assessed by linear regression analysis and correlation coefficients (r). A p value < 0.05 was considered significant. Associations between SHBG and hormone concentrations were assessed with the results reported as coefficient and 95% confidence interval (CI). Analyses were performed using Statistica version 13.1 (StatSoft, Poland).

Results

All the participants were of Caucasian ethnicity. They were first pregnancy planners aged 28–40 years. All reported as being unable to conceive despite at least 12 months of unprotected sexual intercourse and were referred by the gynaecologists for infertility treatment.

The participants denied incidence of menstrual irregularities or missed periods both at present and in the past. All were otherwise healthy and not obese. Thirteen (86.6%) participants revealed normal body mass index (BMI), ranging between 19.1 and 24.8, and 2 of them (13.4%) were overweight (BMI 25.8 and 26.3).

Sperm concentrations of their partners varied between 16.5 and 23.0 million per millilitre of ejaculate, with normal morphology and motility, considered normospermia.

Certain characteristics of the examined patients and steroid hormone concentration ranges during their menstrual cycles are presented in Table 1. Serum concentrations of P4, T, LH, and TSH were within normal cycle reference intervals [7, 8]. The E2 and FSH were higher, whereas SHBG levels exceeded reference intervals most profoundly.

Through all the examined cycles P4 daily mass concentrations prevailed quantitatively over the remaining steroid hormone concentrations. Daily levels of P4 (ng/mL) were between 9.75- and 14.6-fold higher than those of

Table 1. *Certain characteristics of 15 subfertile patients, ranges of their hormone concentrations at baseline (cycle day one), and through cycles vs. reference intervals from menstrual cycles of normal women*

Age [years]	28–40	
BMI [kg/m²]	19.1–26.3	
Cycle length [days]	23–28	
Menarche age [years]	10–12	
Progesterone [ng/mL]		**Reference intervals**
Baseline	0.30–0.40	0.15–0.70 (follicular phase)[a]
Through cycles	0.39–38.4	2.0–25 (luteal phase)[a]
Testosterone [ng/mL]		
Baseline	0.10–0.78	0.06–0.79[b]
Through cycles	0.04–2.63	0.03–1.35[b]
Oestradiol [pg/mL]		
Baseline	23.0–213	20–150 (early follicular phase)[a]
Through cycles	24.8–1010	40–750 (late follicular phase)[a]
SHBG [nmol/L]		
Baseline	26.4–137	0.60–0.97 (follicular phase)[a]
Through cycles	49.0–>180	53.9–78.7 (luteal phase)[a]
FSH [IU/L]		
Baseline	3.4–14.0	1.9–9.9 (follicular phase)[a]
LH [IU/L]		
Baseline	3.5–12.6	1.7–15.0 (follicular phase)[a]
TSH [μIU/L]		
Baseline	0.8–3.8	< 4.0 (cycle unrelated, manufacturer)

SHBG — sex hormone binding globulin; FSH — follicle stimulating hormone; LH — luteinizing hormone; TSH — thyroid stimulating hormone.
To convert ng/mL into nmol/L multiply by 3.18 for progesterone and by 3.46 for testosterone. To convert pg/mL into pmol/L multiply by 3.67 for oestradiol.
Reference sources: [a][7], [b][32]

T (ng/mL), and between 16.25- and 38.01-fold higher than those of E2 (pg/mL). In turn, daily T concentrations prevailed over E2 concentrations by between 1.61- and 2.60-fold.

The daily concen1trations of P4 correlated negatively with T concentrations, and T correlated negatively with E2 concentrations. In turn, P4 daily concentrations correlated positively with E2 concentrations during both the entire cycle length and within their halves (Tab. 2).

Figure 1 shows that the mean ± SEM daily concentration curves of P4 and E2 rose and fell in parallel from the cycle beginning until its end. They were overlapped since cycle day 18, culminated closely, and decreased in parallel thereafter.

The mean daily levels of P4 attained the ovulation threshold serum level of ≥ 5 ng/mL [9] from day 12 onward, which preceded by 2 days the maximal daily level of E2 (day 14), a surrogate index of ovulation. When applying another ovulation threshold level criterion of P4 ≥ 9.54 nmol/L (3.0 ng/mL) [10], the mean daily ovulation threshold of P4 was attained earlier, by cycle day 10.

When percentage changes were calculated in relation to cycle day 1 (100%), the mean P4 daily level increased to 1300% on day 14 and to 2571% on day 16. In turn, the mean daily levels of E2 increased to 580% on day 14 and to 520% on day 16.

The averaged daily concentrations curve of T levels was the opposite. The highest T levels were revealed at the cycle beginning (normal levels according to reference interval) between days 1 and 4. Then T concentrations steadily declined, with a nadir (–27%) on day 16, concomitantly with the maximal daily concentrations of P4. The T curve stepwise recovered to 94.6% of the averaged baseline daily levels on day 28 (Fig. 1).

Daily levels of SHBG were positively associated with E2 levels (β = 0.32, CI: 0.23; 0.42, n = 392) and negatively with T levels (β = –0.26, CI: –0.36; –0.17, n = 392), and were not associated with P4. Because of the wide range of individual SHBG daily level variations, the cycles were divided into shorter, lasting 23–26 days (n = 7), and longer, lasting 27–28 days (n = 8).

The menstrual pattern of the averaged daily levels of FEI in shorter cycles was lower overall, irregular,

Table 2. *Correlation coefficients (r), their significance (p), and the number of pairs correlated (n) between progesterone and testosterone (P4/T), between T and oestradiol (T/E2), and between P4 and E2 (P4/E2) daily levels through the entire menstrual cycles or within their halves (days 1–14 or days 15 till cycle end)*

	Entire cycle			Days 1–14			Days 15 till cycle end		
Pairs	**r**	**p**	**n**	**r**	**p**	**n**	**r**	**p**	**n**
P4/T	–0.13	< 0.05	391	0.12	NS	210	–0.11	NS	181
T/E2	–0.19	< 0.05	391	–0.08	NS	210	–0.28	< 0.05	181
P4/E2	0.38	< 0.05	392	0.39	< 0.05	210	0.52	< 0.05	182

NS — non-significant

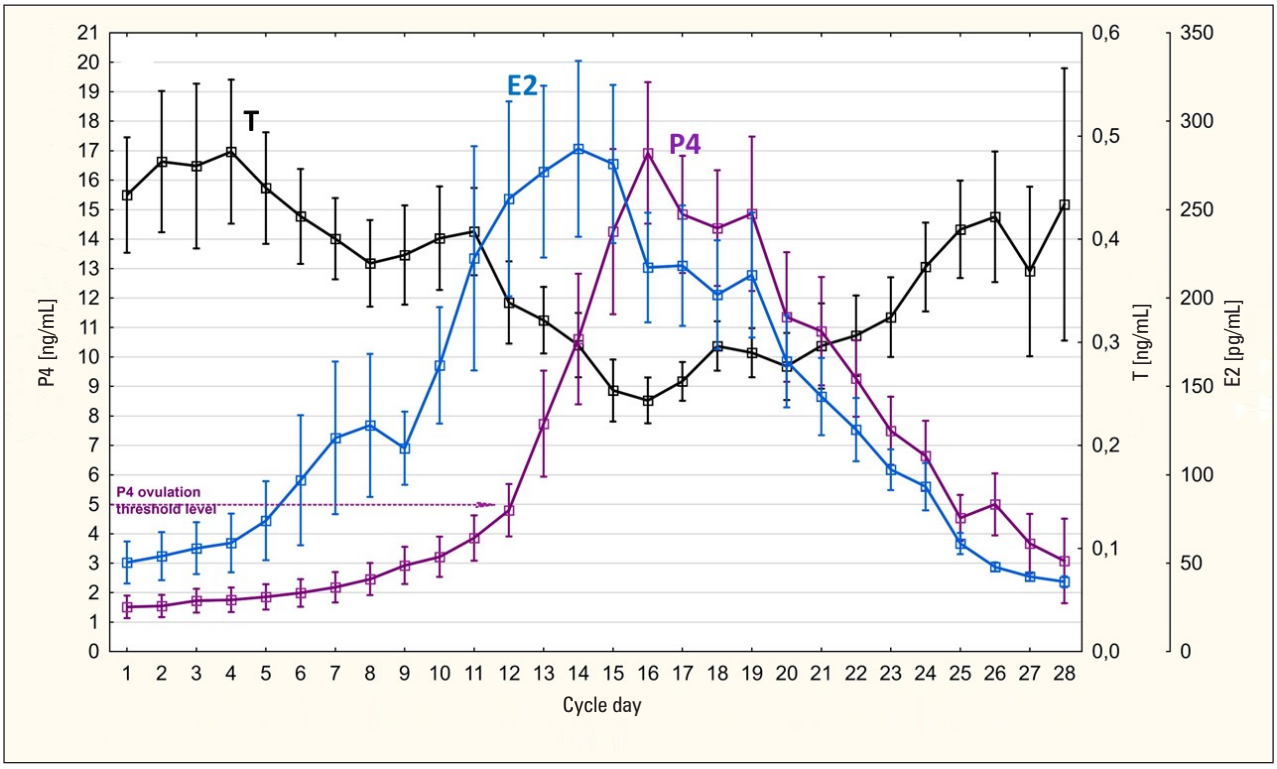

Figure 1. *Curves of the mean ± standard error of the mean (SEM) daily serum levels of progesterone (P4) (violet), total testosterone (T) (black), and total oestradiol (E2) (blue), during menstrual cycles in eumenorrheic subfertile women. Median values of the examined days were significantly different along the curves (ANOVA). Dotted arrow indicate the point of an achievement by the mean daily P4 of the ovulatory threshold level of ≥ 5 ng/mL. Note the same units for P4 and T mass concentrations (ng/mL), and 1000-fold lower units for E2 (pg/mL)*

and flattened with respect to those of 27–28-day cycles. In shorter cycles the maximal daily FEI level was located in day 11, whereas in longer cycles in days 14–15. In shorter cycles significantly higher FEI levels were found on day 7 and lower on days 15 and 16, comparing to longer cycles (Fig. 2). The curves of the averaged daily levels of FAI did not differ with respect to the cycle length (data not shown).

Discussion and Conclusion

To our knowledge, this is the first study that provides daily measurements of 3 main ovarian steroid hormones simultaneously throughout the entire length of the natural menstrual cycles in normally menstruating subfertile women.

Menstrual cycle patterns of the ovarian hormones are indicative of ovarian follicle maturation (represented by E2 rise and mid-cycle culmination in the follicular phase), followed by follicle luteinisation (represented by P4 rise in the luteal phase). Our study shows that in subfertile women the expected menstrual cycle phases are hidden or fused whereas P4 secretion is quantitatively predominant through the entire cycle length.

Specifically, the predominance of P4 could be shown by 1) a premature rise in P4 secretion, identified as precocious achievement by P4 of an ovulatory threshold level. 2) The highest amplitude of P4 rise, more than 4-times greater than the amplitude of E2 and associated with a decline in daily T levels. An ovulatory threshold level of P4 is a hormonal parameter of clinical significance for the prediction of passed ovulation. It occurred in our study since cycle day 10 or 12, depending on the criteria used, sooner than those reported for normal women (days 16–17, [10]).

We have demonstrated for the first time that when the menstrual patterns of P4 and E2 were rose and fell in parallel, the pattern of T steadily declined. A pathophysiological basis for the observed convergence would be a common tissue source of P4 and E2 secretion, the granulosa cells. The observed divergence would be facilitated by a separate source of T secretion, the neighbouring theca cells [6, 11, 12]. A parallel patterns of the serum P4 and E2 levels, restricted to the luteal phase, could be deducted from an earlier study in normal women [13].

A slight premature rise of P4 (> 1.5 ng/mL), viewed on a single day of the cycle, has been shown to produce lowered implantation and pregnancy rates [2, 3]. It can-

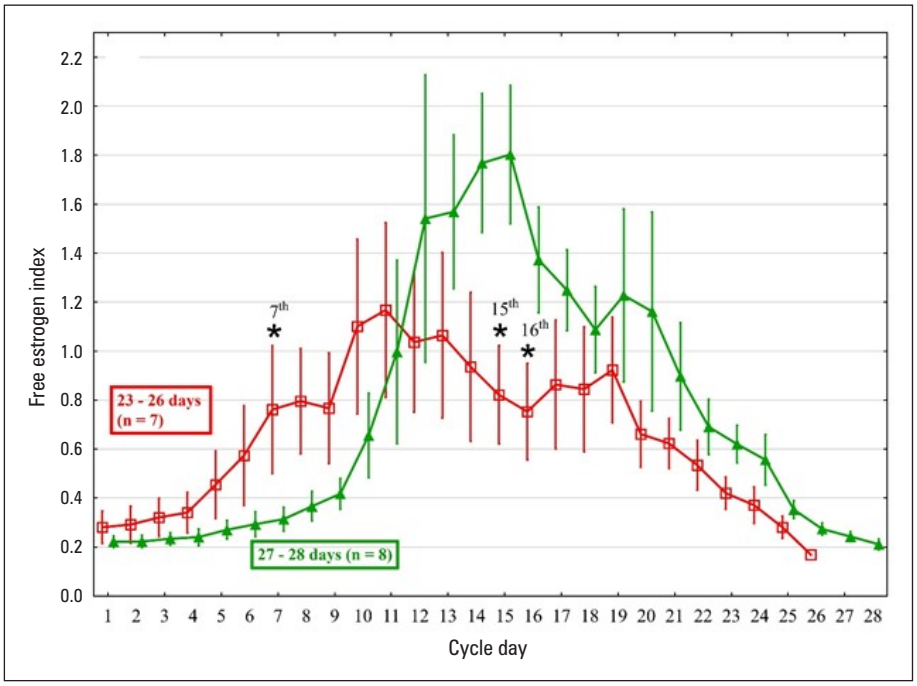

Figure 2. *Curves of the mean ± standard error of the mean (SEM) serum daily levels of free oestrogen index (FEI) through menstrual cycles, divided according to cycle length. Asterisks indicate significant differences (ANOVA) between shorter and longer cycles*

not be excluded that a similar gene expression pathology may occur in natural cycles of subfertile women where P4 rose prematurely and culminated with a huge level. This remains to be further studied.

Correlations between the individual daily hormone levels showed that P4 was inversely related with T, and that the same concerned T and E2 levels. Both correlations are suggestive of intact substrate-product relationships between hormones in the ovarian steroidogenic pathway. An imbalance where the highest P4 levels coincided with the lowest levels of T was observed on day 16. Less expressed dissociation between P4 and T secretions can be deducted from the study, based on the follicular fluid content, aspirated following an LH surge in normal women [11, 14], indicative of ovarian follicle luteinisation. Numerous premature luteinisations are associated with infertility [2].

The observed P4/T imbalance might be facilitated by a direct inhibitory effect of the augmented P4 secretion on the biosynthesis of T. It has been shown that in healthy women oral contraceptive drugs containing P4 directly inhibited circulating T, before presentation of their negative feedback exertion on LH secretion [15]. Administration of E2 also directly inhibited ovarian T production via Cyp17al expression in adult rats [16].

Observed progressively declining levels of T from the cycle beginning until the mid-cycle nadir might be facilitated by utilization of T as a substrate for the biosynthesis of E2. Although the activity of aromatase, an enzyme that converts androgen to oestrogen, did not follow E2 raise during menstrual cycle [17–19], Sano et al. [18] and Doody et al. [20] postulated that E2 biosynthesis may be regulated predominantly by the supply of the substrate for aromatization rather than aromatase activity. To explain a partial recovery of daily serum T concentrations from the mid-cycle region until the cycle end, it is important to note that the fluid of follicles punctured following LH ovulatory surge in normal women is rich in the atretic follicles containing androgens [11, 21].

The observed U-shaped pattern of T levels through the menstrual cycle has never been described in healthy women. While some authors denied the existence of menstrual cycle-dependent fluctuations in serum T [22], most of the others reported its cyclical variation with the highest values in the mid-cycle periovulatory period [8, 23–26] or in the cycle beginning and mid-cycle regions together [27, 28]. Differences in the magnitude of hormone changes, methods of cycle alignment, frequency of sample collection, and difficulties recruiting participants that yielded homogeneity regarding their cycle length might account for the inconsistency. Above all, when the common finding was that the maximal T levels occur in the mid-cycle region in normal cycles, our results are in contrast to those, and hence a suboptimal menstrual cycle profile of T secretion in subfertile women may be postulated.

Menopause may occur in women of any age, but crucial symptoms are menstrual irregularities and missed periods. Neither of these symptoms were reported by our participants. However, a moderate 20% decrease in circulating T was described in healthy women long before menopausal transition, beginning from the age of 25 years [29]. This age-dependent decline in the ovarian androgen production might be represented by our subfertile participants.

Although an increased serum T is associated with increased incidence of polycystic ovary syndrome and infertility [30–32], the low functional ovarian reserve and the primary ovarian insufficiency in infertile women are acclaimed to hypoandrogenaemia [33]. It has been shown that T plays physiological role during follicular development [34, 35]. Rice et al. [36] demonstrated that human early preantral follicles involve T activity before they acquire FSH receptor during further development. In addition, both natural follicular atresia [37] as well as luteolysis [38] have been acclaimed to the presence of T. These findings may imply that a constant availability of T through the entire menstrual cycle is mandatory, and the observed relative deficiency in T secretion may be harmful, contributing to the pathogenesis of subfertility.

Exogenous E2 was shown to stimulate SHBG production in postmenopausal women [39], but SHBG levels were reported to remain constant during normal menstrual cycle or increase in its second half [8]. In our study, no cycle-dependence was noted; however, the individual SHBG daily levels were positively associated with E2, and negatively with T levels. This indicates that the hepatic SHBG synthesis can be modified by the ovarian function. On the other hand, among the 2 calculated indexes of the hormone bioavailability (FAI and FEI), only the FEI pattern revealed significantly different daily levels during the cycles shorter than 26 days. This suggests that SHBG may actively participate in determination of the menstrual cycle length by modulating E2 bioavailability.

Summing up, the presented study indicates that in subfertile women the ovarian steroid hormones interrelate with each other along the entire menstrual cycle length with an intact steroidogenic pathway. The following changes appear to be most characteristic: 1) Throughout the entire menstrual cycle length the P4 secretion predominates quantitatively over the secretions of the remaining sex hormones when menstrual cycle phases are hidden. 2) A rise in E2 secretion is in parallel with a rise in P4 but with 4-times lower amplitude of E2. 3) T secretion declines and is inversely related to both P4 and E2 secretions. 4) Changes in E2 bioavailability are related to the menstrual cycle length. An enhanced ovarian steroidogenesis and/or the functional imbalances in the steroidogenic pathway may be associated with subfertility in normally menstruating women. It must be anticipated that the medical challenge against the augmented ovarian steroidogenesis [40] may have a therapeutic significance in the preconception management for subfertile women. However, further studies are needed to increase the clinical material and the amount of natural cycle sampling in both fertile and subfertile women.

Authors' contributions

K.K. — the concept, project design, literature search, interpretation of the results, and manuscript writing; B.C.A. — project design, patient enrolment, supervision of hormone measurements, crude data presentation, and funding acquisition; S.B. — statistics, data processing and figure, and table preparations; O.W.K. — patient management and editorial assistance; M.K. — manuscript final adjustments.

Institutional Review Board Statement

The study protocol was approved by the Bioethics Committee of the Medical University of Lodz (No. RNN/226/16/KE of 12 July 2016).

Data availability

The datasets generated and analysed during the current study are available from the corresponding author upon reasonable request.

Competing interests

BCA is a director of Gyncentrum Katowice. The remaining authors declare no competing interests.

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