Changes in serum testosterone during the menstrual cycle – an integrative systematic review of published literature

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ABSTRACT

The effect of sex hormones in the pathogenesis of many diseases such as rheumatoid arthritis, systemic lupus erythematosus and asthma has been highlighted in addition to their role in reproductive physiology. Among the sex hormones, the effects of testosterone (T) in females constitute an important area of research. The present review critically evaluates the published literature on serial T measurements during the menstrual cycle (MC) in healthy females. Articles describing serum T measurements during the MC were identified by searching the PubMed database in October 2020. The keyword combinations used for the search were (testosterone OR androgen) and (menstrual cycle OR ovarian cycle). The literature search yielded a total of 1009 articles and five additional articles were identified by screening reference lists. A total of 67 articles were eligible to be included in the final analysis as per the inclusion and exclusion criteria. Serum T level was found to be low in the early follicular phase and the whole of the luteal phase, demonstrating a surge at the time of ovulation, irrespective of the study methodology, the techniques used to assess T, and the age of the women studied.

KEYWORDS

Testosterone, androgen, menstrual cycle, ovarian cycle.

Introduction

Androgens in the female reproductive system

In women of reproductive age, dehydroepiandrosterone sulfate (DHEAS), dehydroepiandrosterone (DHEA), androstenedione, testosterone (T) and dihydrotestosterone (DHT) are found in the circulation, but only testosterone and DHT are able to activate androgen receptors. DHEAS, DHEA and androstenedione are considered prohormones, requiring conversion into T or DHT to express their androgenic effects [1].

Testosterone

Testosterone is a steroid hormone ($C_{19}H_{28}O_2$), and women have lower levels than men. The importance of estrogen and progesterone in female reproduction and chronic disease is well documented but the role of T in women's health is less emphasized [2]. In females, serum androgen levels are higher than serum estrogen levels, even though the main effects of these hormones in women are those mediated by estrogen. It is reasonable to assume that T, too, has important physiological effects in women[3, 4] .

Research on androgens in women has focused almost exclusively on issues of excess, and it was recently shown that androgens play an important role in the health and well-being of women[5]. Testosterone is known to maintain muscle mass and bone strength, enhance sex drive, and facilitate an overall sense of well-being and zest for life in women.

Yet the effects of T and its action on inflammatory conditions like asthma are not clear. Much of what is known comes from the study of conditions of excess or insufficiency in men and women, or from extrapolation of results from *in vitro* and animal studies [3].

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Production of testosterone in females

In females, approximately 0.02 micromoles of T is secreted per day from the theca cells of the ovaries and the adrenal cortex [6]. Peripheral conversion of androstenedione (prohormone) in adipose tissue accounts for about half of the circulating T [7]. From birth to puberty there is no significant change in the level of T in females[8]. After puberty, increased peripheral conversion and increased production leads to higher T levels [9, 10]. Ovarian synthesis of testosterone occurs in theca cells and is mainly regulated by luteinizing hormone (LH) [11] and also by insulin[12]. Testosterone is then transported to the preovulatory granulosa cells, where it is aromatized to estrone and estradiol by 17-β-hydroxysteroid dehydrogenase type I, stimulated by Follicle stimulating hormone (FSH) [13]. Ovarian androgen production and conversion of T into estradiol are essential to the physiological process of ovulation and insufficient androgen production in the follicular phase can lead to anovulation [14] .

Measurements of serum testosterone

In females only 1% of T remains free and the rest is bound to proteins like sex hormone binding globulin (SHBG), albumin and cortisol binding globulin, reducing its bio-availability [7]. In ascertaining serum T levels, it is necessary to measure both the free testosterone (FT) and the albumin bound fractions. The 'free testosterone index' (FT level/SHBG level) is used for FT measurement in females[15]. The serum total testosterone (TT) level exhibits a circadian rhythm, with the highest observed in the early morning and the lowest in the late evening [16, 17]. Hence, serum T levels need to be interpreted with caution.

Total testosterone can be measured through immunoassays (IA) or by mass spectrometry (MS), which is considered the gold standard [18, 19]. Immunoassays can be performed either directly by radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), fluorescent immune assay (FIA), chemiluminescence immunoassay (CLIA), electro chemiluminescence immunoassay (ECLIA) or by means of each of these coupled with extraction and/or chromatography [18-20]. The extraction and/or chromatography procedures remove interfering proteins and increase measurement sensitivity[18].

Krakowsky *et al*. showed higher levels of T on ECLIA compared with liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) method, which is probably the most appropriate for measuring low testosterone levels[21]. FT can be measured indirectly by equilibrium dialysis (EqD), directly by using an analog-based RIA (analog FT), or through standardized calculations. Equilibrium dialysis is costly but remains the gold standard in measuring FT[18].

Most current methods do not measure T levels in women accurately [22]. Measuring serum T levels has limitations due to issues with the method accuracy, precision, sensitivity and specificity at the low hormone levels found in women. Understanding of the clinical significance of T across the menstrual cycle (MC) has remained limited, partly due to these factors. Body mass index (BMI) [23], female smoking, alcohol consumption and exercise influence T levels in females [24].

Testosterone and chronic disease

In recent years, the role of T in the pathogenesis of asthma, rheumatoid arthritis, systemic lupus erythematosus (SLE), endometriosis, systemic sclerosis, and multiple sclerosis in women has been identified [25-32]. Indeed, low T has been linked to the pathogenesis of asthma [25], SLE [28], rheumatoid arthritis [30], and type 2 diabetes[33]. Testosterone supplementation has been found to be useful in chronic heart failure and to improve insulin resistance in elderly women [34]. The anti-inflammatory properties of T are explored as a probable disease-modulating mechanism in the above chronic inflammatory diseases. Studies demonstrate that T reduces TNF-α, interleukin isoforms, and C reactive protein (CRP), by various and often overlapping physiological mechanisms [35].

With the aim of establishing the current level of knowledge in this field, an in-depth critical analysis of the published literature was conducted to assess the variations in T levels throughout the MC.

Methodology

The authors performed an integrative systematic review of published literature, indexed in the PubMed database, describing changes in T levels during the MC in otherwise healthy females.

Database search

Studies reporting fluctuations in serum T levels during the MC were identified using the PubMed database (U.S. National Library of Medicine). The search included all relevant articles published up to October 2020. The PubMed database was searched using the terms 'testosterone' OR 'androgens' AND 'menstrual cycle' OR 'ovarian cycle' in a 'title', 'abstract' or 'keywords' search. Full articles of the included studies were accessed through the University of Sri Jayewardenepura.

Search method

Articles identified from the keyword search were transferred to an Endnote database. Once duplicates were removed, the title, keywords and abstracts were reviewed for suitability for inclusion. All full texts except for 18 were accessed and reviewed for suitability (considering gender and age group of samples, and reporting of T levels). Forward citations of the studies retrieved were also traced and screened for possible inclusion and five additional articles were identified for inclusion. The search was conducted independently by KA and LA and the articles to be included in the review were determined through an interactive consensus process involving KA, NS, LA and DMSF (Fig.1).

Inclusion and exclusion criteria

For ease of comparability, the review included only research articles published in English and referring to serum samples, even though sputum or salivary T levels are considered good surrogates for serum T. Conference proceedings, editorials, commentaries, book chapters and book reviews were excluded. Studies describing two or more serum T readings across the MC were included even though such measurement was not the main objective of those selected studies. Studies that did not mention T values separately for each phase of the MC were excluded.

Data extraction

The full texts of the selected articles were reviewed and relevant variables were extracted into a datasheet. Extracted variables included, author/s, year of publication, method of detection of serum T, sample size, demographics of the sample, sample collection method, MC phase in which the sample was collected, and T values recorded.

Results

The primary search identified 1009 articles which were reviewed for duplicates, after which the title, keywords and abstracts of the remaining articles were reviewed. Five additional articles were identified by manually searching the reference lists and forward citations of included papers. A total of 67 articles were included in the review. Tables 1 and 2 summarize the studies included in the review.

Figure 1 Flowchart for the study selection process.

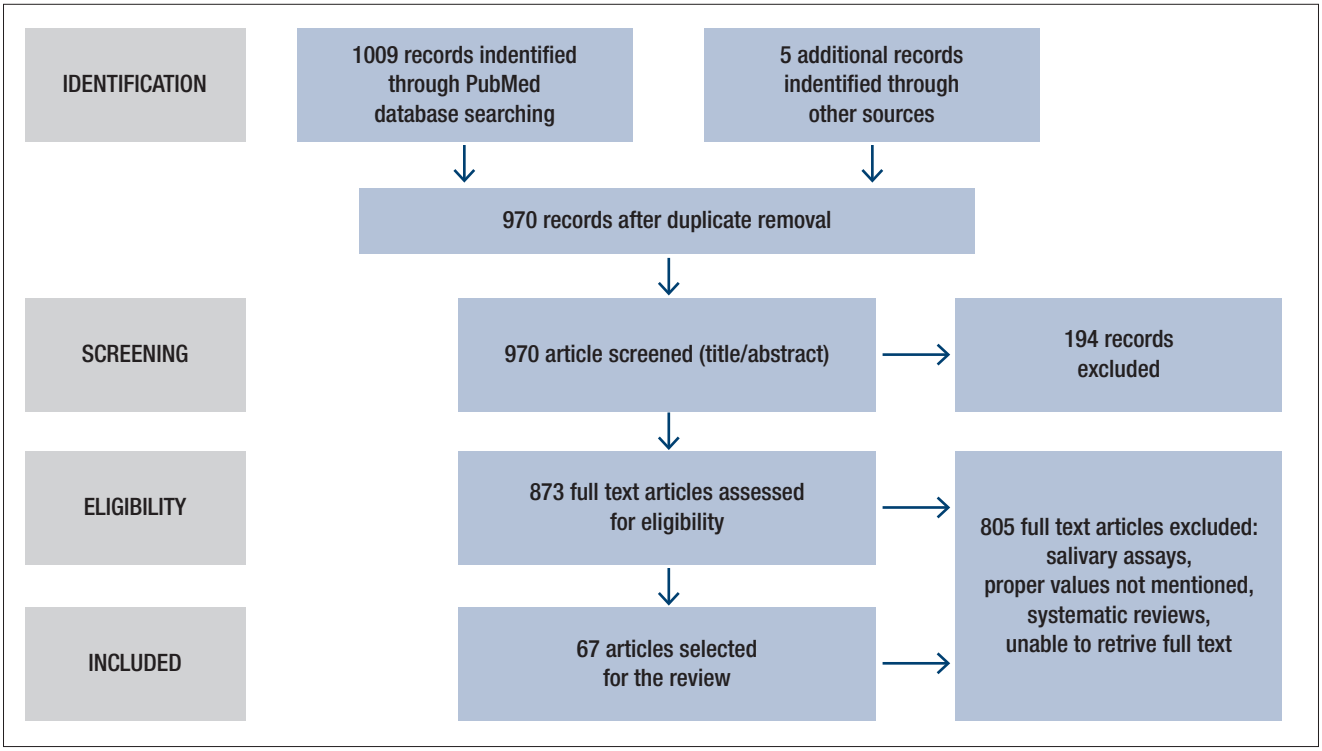

Table 1 Summary of results of the studies reviewed.

AUTHOR	METHOD	DEMOGRAPHICS AND SAMPLE SIZE	BLOOD COLLECTION TIME	TIMING IN THE MENSTRUAL CYCLE	MAIN FINDINGS
Bui *et al.* 2013[36]	T was measured by ID-LC-MS/MS) and compared with CMIA	25 regularly menstruating females and 44 PCOS females	SC early morning	Daily mean serum T concentrations (~28 points) in normal females	Mean T was higher in the mid cycle p<0.05(with both methods). The mid-cycle elevation of T may not be clinically relevant since day-to-day variation is higher and independent of the MC.
Goebelsmann *et al.* 1974[38]	Serum T level was measured by RIA	8 healthy females with RMPs	8-10 am	Daily ~28 points	Highest T level was observed on the day of LH peak (p<0.005)
Judd *et al.* 1973[39]	Serum A and T were measured by RIA	6 females with RMPs	8-10 am	Daily for 28 days	The mean A, and T level during the middle third of the MC was significantly higher
Oka *et al.* 1988[40]	HPLC was compared with RIA method	6 healthy females with RMPs	BSC at 11.00am	Twice during days 4 to10 and twice during days 20 to 26	T was significantly higher during LP than FP when determined by HPLC method but not by RIA.
Nobrega *et al.* 2009[43]	TT was measured by RIA	8 healthy females with ovulatory RMPs	BSC between 7-8 am	During day 7, day 12, day 21 in 3 consecutive MCs	No statistically significant difference in the mean values of the parameters between 3 MC phases.
Matteis M *et al.* 2014[46]	TT was measured by ECLIA	36 asthmatic females, aged 23-43 years	8-10 am	At 2 points in the MC: mid FP and LP	TT was higher during the LP
Edam *et al.* 2019[48]	TT was measured by ELIFA	70 healthy females and 70 patients with bronchial asthma	Not available	At 2 points in the FP and LP	T was significantly elevated during the LP in both asthmatics and controls. p<0.05
Sramkova 2015[49]	TT was measured by RIA	27 females with regular menstrual cycles	7-8 am	Every 3rd day starting from day 1	There was an increase in T during ovulation
Shultz *et al.* 2006[51]	T was measured by chemiluminescence assays	22 females (18-30 years) normal menstrual cycles	8am-noon	Daily	Highest level TT was seen post ovulation
Delale *et al.* 1998[52]	T levels were measured by RIA	Six parous and four nulliparous females	Not available	On days 2-4, 6-9, 11-14 and days 1-3. 6-9 after the LH surge	Highest level of TT was recorded during ovulation followed by LP, early FP and late FP
Ahrens *et al.* 2014[53]	Serum TT was measured by LC -MS/MS	259, healthy premenopausal females	Not available	On day 2, day 7, days 12/13/14, and days 18/22/27	Highest level of TT was recorded during ovulation

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AUTHOR	METHOD	DEMOGRAPHICS AND SAMPLE SIZE	BLOOD COLLECTION TIME	TIMING IN THE MENSTRUAL CYCLE	MAIN FINDINGS
Apter *et al.* 1978[55]	Serum T was measured by RIA	20 girls aged (13-17 yrs) a. 10 girls 2.9 years after menarche b. 4 girls 1.5 years after menarche, 6 girls 1.1 years after menarche	9.30-10.30 am	On days 1-3 and every 2nd day thereafter during first 10 days: every day during next 10 days: then every 2nd day until next menstrual bleeding	Girls in group 'a' had a periovulatory rise in T in the mid cycle. No periovulatory rise in T was seen in group 'b'. In group 'c' (all anovulatory cycles), serum level of T was significantly higher during FP than in group 'a' or 'b'.
Bancroft *et al.* 1983[56]	TT method not given	55 women with normal ovulatory MCs	Not available	Not available	TT increased significantly during middle one third of the cycle especially in the mid cycle
Brideau *et al.* 1992[59]	TT was measured by RIA	28 females BMI: 27kg/m^2:	Fasting BSC in morning	From day 2 of MC to day 1 of next MC	A small increase in TT during mid cycle
Dougherty *et al.* 1997[61]	TT was measured by RIA	Twelve females, aged 18-39, (mean 24.8 (SD 7.5)	Not available	At 4 points (menses, mid FP, ovulation, and pre-menstruation)	TT peak during ovulation but similar in all other phases. Within subject changes in TT levels vary greatly
Epstein 1975[62]	Plasma TT was measured by RIA	9 females with RMP; mean age 34 years (range 25-43), mean weight 57.2 kg	8.30-10 am	Daily for one to two consecutive cycles	Mean T level was significantly higher during the 7 days before and after the mid-cycle LH peak when compared to the premenstrual phase - 4 days before menses (p<0.0l)
Frolich M *et al.* 1976[63]	TT, A, DHEA were measured by RIA	7 healthy females with RMPs	9-11 am	Daily, during 6 normal cycles	A, TT significantly elevated from 3 days before until 3 days after ovulation
Genazzani *et al.* 1983[64]	TT, DHA, DHT were measured by RIA after plasma extraction, A was measured by RIA	a. 5 menstruating girls 14–17 years, b. 6 females 20-25 years, c. 6 females 23-27 years	Not available	a. Every 2-4 days from day 5 b. Daily but EOD during early FP and late LP c. Days 5-6, 13-14, 23-24	A and TT showed a progressive significant increase to reach the maximum levels which for A, DHA occurred concomitantly with O peak and for TT 24 hours later
Guapo *et al.* 2009[65]	TT was measured by RIA	30 healthy females with RMPs aged 18–29 years (mean± S.D.-22.1 ± 2.9)	Not available	From either early FP (days 1-5), ovulatory (days12-14) or LP (days 21-23) of cycle)	TT varied significantly. Highest level during ovulation, followed by early FP and LP. DHEAS, A showed no change. SHBG increased from mid FP to ovulatory phase, peaked during mid LP, with a significant variation from mid FP
Kim *et al.* 1976[66]	TT was measured by competitive protein binding method	4 healthy females, 19-28 years with RMPs	8-10 am	Daily except for early FP (days -14 to -10) and mid LP (+5 to +9)	TT level of the mid cycle period was significantly higher than that in early FP and mid LP
Kurzer *et al.* 1986[67]	TT was measured by RIA A, DHEAS, SHBG (solid phase method)	6 healthy females 19-29 years with RMPs	EOD	Fasting BSC in mornings	TT peaked during mid cycle, being higher in the late FP and periovulatory phase than in EFP and LP. A also peaked mid cycle. DHEAS, SHBG showed no difference.
Rubinow *et al.* 1988[70]	TT, DHT were measured by RIA	9 females with RMPs	Fasting BSC in mornings	During days 1,5,10,15,18,21, 23, 25, 27	TT and DHT changed significantly through MC. TT highest during late FP and decreased gradually
Engel *et al.* 1981[72]	TT was measured by RIA	30 females 20-30 years.	between 8 and10 am	At 3 points: early FP (days 4-6), ovulatory (- 10-16 reverse cycle), mid LP (days 4-7 reverse cycle)	Mean TT during the ovulatory phase was significantly higher than during FP, LP (p<0.05). No significant difference found between FP, LP
Engel *et al.* 1989[73]	TT was measured by RIA	17 females with hypoactive sexual desire disorder and 13 controls aged 21-45 years	8-10 am	Every 3, 4 days during MC	TT displayed significant differences (p < 0.0001) with peak values occurring during the periovulatory phase. FT did not differ between the phases
Van Goozen *et al.* 199774	TT, DHEAS, A measured by RIA, FAI by calculation	21 healthy females (mean 29 years): 11 with and 10 without premenstrual complaints	7.30-8.30 am	Three times per week at 2-day intervals	TT changed significantly with a peak half way through the cycle with corresponding lower values of SHBG. Calculated FAI increased at mid cycle
Vermeulen *et al.* 1976[75]	TT, DHT, A, DHEA were measured by RIA	15 females (18-23 years) with RMPs	8-9 am	Daily	Mean T, A, DHT were significantly higher around ovulation than in LP, FP. Clear peak was seen only for A. No change in DHEA across MC
Duskova *et al.* 2012[76]	Conjugated DHEA, A, T, 5α-DHT were measured by GC-MS	10 females in follicular phase and 10 females in luteal phase	Not available	3rd-5th day of MC in the FP and 22nd-24th day in the LP	A, TT in FP were higher than in LP. FT and FT index were both higher during the LP than in the FP.

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AUTHOR	METHOD	DEMOGRAPHICS AND SAMPLE SIZE	BLOOD COLLECTION TIME	TIMING IN THE MENSTRUAL CYCLE	MAIN FINDINGS
Okifuji *et al.* 2006[77]	TT method not given	74 healthy females below 40 years RMPs	Not available	During 3 points in MC: Mid LP (+5 to +9 day from the ovulation), Perimenstrual phase (-2 to +2 days, counting from menstrual onset), Mid-late FP (-1 to -5days, counting from the ovulation)	T during mid FP was higher than that during the mid-LP ($p < 0.004$)
Hill *et al.* 2005[79]	TT, DHT, DHEA were measured by GC-MS	Females 22-45 years of age with RMPs: 15 women in FP, 17 women in LP	Not available	During FP and LP	Females in the LP of the MC had higher levels of T, DHT, DHEA
Kannenberg *et al.* 2018[80]	TT was measured by CLIA & GC-MS, DHT by EIA	(23-43 years) 81 females with RMPs free of chronic diseases	8am-noon	During FP (days 3-5) and in LP (days 21-23)	Higher TT and DHT seen during the LP
Hietala *et al.* 2007[82]	TT was measured by ECLIA	258 young menstruating Caucasian females	Not available	Once on days 5-10 and once on days 18-23.	T was higher during the LP than in the FP (1.65 versus 1.45 nmol/l; $p = 0.0001$)
Salonia *et al.* 2006[83]	TT was measured by ECLIA	47 Caucasian healthy females with RMPs	8-10 am	Days 5–8; n = 24 or days 19–22; n = 23	TT, FT, SHBG levels were higher during LP. DHEAS was lower during LP.
Anttila *et al.* 1991[84]	T was measured by RIA, A by RIA, DHEAS was measured by RIA, SHBG was measured by ligand binding assay	40 healthy females with RMPs aged 19–40 years (32.8 ± 5; mean S.D.) BMI 18 to 35 kg/m^2) (23.3 ±3.5)	7-9 am	At 2 points in the early FP (days 3-7) and in the mid LP (days 22-24) of MC	The mean levels calculated for T and SHBG were significantly higher ($p = 0.0004$ and $p = 0.0001$, respectively) in LP; no differences were found in other androgen values.
Apter *et al.* 1976[85]	TT was measured using chromatography, RIA	Healthy females RMPs	Before noon	During FP and LP	T, DHT were significantly higher during LP
Skiba *et al.* 2019[86]	TT was measured by LC-MS/MS	Caucasian white females: 163 in the early FP,184 in mid cycle, and 241 in LP	Not available	Either from early FP (day 1-7), in mid cycle (days 8-21), and in LP (days 22-35)	TT and A varied across MC with statistically significant nadirs in the early FP compared with the mid cycle and LP
Jackson *et al.* 202087	TT was measured by 125I-based ImmuChem double antibody	20 females (n = 11) during FP and (n=9) during LP	7 am-noon	Single blood sample taken during either FP or LP	T was similar during the LP and FP of the MC
Arnoni *et al.* 2017[88]	TT, DHEAS were measured by ELISA	20 females with RMPs and 12 taking OCP	Fasting BSC	BSC at 2 points	NO difference in T between FP and LPs
Collins *et al.* 198590	TT was measured by RIA	15 women aged 21-36 years (mean 29.5 years) with RMP	Not available	At 3 points during MC: FP, ovulatory phase and LP for 2 consecutive MCs	TT did not show any cycle-related changes.
Dada *et al.* 1984[91]	TT was measured by RIA	17 apparently healthy Nigerian females aged 20-25 years with RMPs	9-11 am	EOD during first 8 days of MC, daily during rest of MC	Mean TT fluctuated rather widely with no discernible pattern in either proliferative phase or LP
Dawood *et al.* 1976[92]	TT and DHT were measured by RIA	20- to 40-year-old 7 healthy females with regular cycles	9-10 am	Daily around mid - cycle and EOD during rest of the cycle	Mean TT rose from early FP to late FP with a drop during ovulation (not significant). After ovulation, the mean TT rose sharply, decreased near menstruation. No variation in anovulatory cycles
Elliott *et al.* 2003[93]	TT was measured by ELIFA	7 healthy females	Not available	During day 2 and day 21 of the cycle	There was no significant difference in TT during the MC
Fahmy *et al.* 1992[94]	TT was measured by RIA	24 healthy females with RMPs; 20-35 years; para 2 to 6; no chronic disease	Not available	In half of the females in mid-FP (day 6-9) half in the mid LP (days 19-22) between 9-11 am	No significant difference in mean T levels across the different phases.
Feldman *et al.* 1978[95]	TT was measured by RIA	11 normal females with regular menstrual cycles	BSC same time each visit to minimize diurnal variation		The level of TT was constant throughout the MC
Luthold *et al.* 1993[96]	TT was measured by RIA after ethyl extraction	Females with RMPs, 28.5 ±1.6 years, with BMI 23.3 kgm^{-2} ± 2.9	Fasting 8-10 am	At 2 points: in FP (days 4–8), in LP (days 14–22)	Mean T concentration was similar in both phases. SHBG level was elevated during the LP.

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AUTHOR	METHOD	DEMOGRAPHICS AND SAMPLE SIZE	BLOOD COLLECTION TIME	TIMING IN THE MENSTRUAL CYCLE	MAIN FINDINGS
Schijf *et al.* 1993[98]	TT was measured by RIA, SHBG was measured by IRMA	56 healthy females with a mean age of 28 (SD 4.7; range 18-35) with a BMI of 22.6 kgm-2 (SD 2.4)	Fasting 8- 9.30 am	In FP (days 9-11), LP (days 21-23)	TT level was unchanged during the cycle. SHBG significantly increased from FP to LP. FAI was significantly lower during the LP.
Schoning *et al.* 2007[99]	TT was measured by immunofluorimetric assay	12 females with RMPs on no medication	2-5 pm	BSC at 2 points: in early FP (days 1-3 days), mid LP (individual cycle length -7days)	TT did not differ significantly between phases.
Tyler *et al.* 1975[100]	TT was measured by RIA	9 females (26-34 years)	8.30-10 am	Daily	No significant change in T, SHBG in the 3 phases.
Valette *et al.* 1975[101]	TT was measured by RIA	8 healthy females (18-32 years) with RMPs	at 9 am, 4 pm, 10 pm	During 3-4 consecutive days at onset (days 3-8), middle (days 11–16) & end of MC (days 23-28)	No consistent pattern of variation of T was seen.
Werawatgoompa *et al.* 19811[02]	TT was measured by RIA	10 females with regular cycles aged 25-33 years	8-10 am	EOD from days 1-9 and daily from days 10-21, EOD till bleeding	No significant difference in T between phases.

TT- total testosterone, **FT**- free testosterone, **FAI**- free androgen index, **A**- androstenedione, **DHT**- dihydrotestosterone, **DHEAS**- dehydroepiandrosterone sulphate, **RIA**- radio immunoassay, **MC**- menstrual cycle , **FP**- follicular phase, **LP**- luteal phase, **EFP**- early follicular phase, **MFP**- mid follicular phase, **MLP**- mid luteal phase, **ELISA**- enzyme-linked immunosorbent assay, EIA- enzyme immunoassay, **LC-MS/MS**- liquid chromatography tandem mass spectrometry, **GC-MS**- gas chromatography mass spectrometry, **HPLC**- high-performance liquid chromatography, **BSC**- blood samples collected, **RMP**- regular menstrual periods, **ID-LC-MS/MS**- isotope dilution liquid chromatography tandem mass spectrometry, **ELIFA**- enzyme linked immunofluorescent assay, **CLIA**- chemiluminescent immunoassay, **CMIA**-chemiluminescent microparticle immunoassay, **ECLIA**- electrochemiluminescence immunoassay, **IRMA**- immunoradiometric assay

Table 2 Summary of the studies that assayed free testosterone.

AUTHOR	METHOD	DEMOGRAPHICS AND SAMPLE SIZE	BLOOD COLLECTION TIME	TIMING IN THE MENSTRUAL CYCLE	MAIN FINDINGS
Massafra *et al.* 1998[37]	TT, FT, A were measured by RIA	12 healthy eumenorrheic females	8-9 am	On days (0= mid cycle estrogen peak) -12, -10, -8, -6, -4, -2, 0, 2, 4, 6, 8, 10, 12, and 14	TT and A levels were elevated during the late FP near mid cycle. FT was increased during late FP and ELP but without a significant peak
Wajchenberg *et al.* 1989[41]	TT was measured by RIA; FT was determined by EqD	10 females with ovulatory cycles and 7 obese women	Not available	BSC at 4 points in MC with mid cycle being considered= Day 0; day -10 to -7 as early FP, -3 to -1 as late FP, +3 to +7 as early LP, +9 to +14 as late LP	In obese females, TT and FT did not change significantly during the various phases of the MC. In normal females, TT was lower at both luteal periods compared with the FP
Veldhuijzen *et al.* 2013[42]	FT was measured by RIA	15 normally cycling healthy females	Not available	During 4 points in the cycle.	No significant MC phase effects were found for T, FT levels
Wyskida *et al.* 2017[44]	TT, FT, A, DHEAS, SHBG was measured by ELISA	52 young, healthy, normal-weight females	8-9 am	On days 2-4, 12-14 and 24-26	TT significantly lower in the FP than both at mid cycle and in the LP of the MC. $p < 0.05$
Rothman *et al.* 2011[45]	T, SHBG and DHT were measured by LC-MS/MS. FT was calculated	31 premenopausal women	Not available	Samples were taken in ovulatory females at 3 points in the cycle. Early FP, mid cycle, mid LP	Average T in mid cycle was approximately 50% higher than in EFP, 22.7 ± 1.7 and LP 15.4 ± 1.6 ng/dl respectively ($p<0.001$). FT was 35% higher than in EFP ($p<0.013$), and did not change from mid cycle to MLP ($p=0.063$)
Linton *et al.* 2016[47]	TT, FT, SHBG were measured by immunoassays	225 females with ovulatory RMPs	Mornings	BSC at 2 points: once in FP and LP	TT and FT were significantly lower in LP than in the FP (both $p<0.0001$)
Wunder *et al.* 2006[50]	FT was measured by automated ECLIA	36 European Caucasian women, 20-32 years, RMPs	6–10 am	BSC from day 1 to ovulation (every 2 days), then, daily until day 19 and every 2nd day during the LP till next menstruation	T was highest during ovulation and decreased towards the end of the cycle
Adams *et al.* 2004[54]	TT was measured by RIA and FT calculated using TT and SHBG	14 females (18-42 years) with RMPs	Not available	Three samples in FP, at mid cycle and in mid LP	TT highest at mid cycle. FT followed the pattern of TT, with a significant effect of cycle phase ($p<0.001$), with the highest levels occurring at mid cycle

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AUTHOR	METHOD	DEMOGRAPHICS AND SAMPLE SIZE	BLOOD COLLECTION TIME	TIMING IN THE MENSTRUAL CYCLE	MAIN FINDINGS
Bloch *et al.* 1998[57]	TT, FT measured by ethanol–ether extraction, chromatography and direct (RIA)	10 females with confirmed absence of premenstrual syndrome (mean age 30.6±5.5 years)	8-10 am	Three times a week for a full menstrual cycle	A significant phase effect was found for T, FT - a periovulatory peak noted. FT was higher during the FP while TT was higher during the LP
Braunstein *et al.* 2011[58]	TT was measured by RIA. Percent FT was measured by EqD using a radiolabeled tracer technique.	161 healthy, Caucasian and Asian American, Hispanic females (18–49 years) with normal cycles	8–10 am	From 107 subjects, samples collected at 3 points: FP (days 5-8), mid-cycle (days 12-14), LP (days 17-21) of the cycle; from 54 subjects, at 2 points: early FP & late LP (D15-28)	Increase in TT corresponding with mid-cycle LH surge was noted. Serum TT approximately 15% higher during the mid-cycle phase compared with the FP. FT increased from the FP to the mid cycle and from the mid cycle to the LP
Caruso *et al.* 2014[60]	TT, SHBG were measured by ELISA. FAI was calculated	1,180 healthy heterosexual females age range 18-40 years	8-10 am	On cycle days 5-7 (follicular phase), 12-15 (periovulatory phase), and 21-25 (LP)	TT, FAI, and SHBG increased from the FP to the periovulatory phase after which they decreased until menses. Significant fluctuations in TT, FT but both peaked during the ovulatory phase
Mashayandebvu *et al.* 1996[68]	TT, FT was measured by RIA. A, SHBG was measured by RIA	2 groups; 7 females (19-37) 7 females (43-47)	Not available	Early FP (cycle days 1 to 2), mid cycle (day of LH surge), and mid-luteal (7 days after LH surge)	The midcycle rise in FT, A seen in younger women was consistently and significantly absent in older women (p= 0.02). SHBG level was constant throughout the cycle
Rezaii *et al.* 2017[69]	TT was measured by both RIA, and LC-MS/MS, and DHEA was measured by ELISA, FT calculated from TT	26 (15 Swedish/11 West Asian) normally menstruating healthy females with an age span of 20–36 years	10 am-noon	At 3 points: in early FP (day 3-5), ovulatory, mid LP (days 7-8 after ovulation)	Significantly higher TT, FT in ovulatory than in FP & LP in Swedish females but not in the West Asian females. SHBG was significantly higher in LP than in FP and ovulatory phase in Swedish females
Salonia *et al.* 2008[71]	TT was measured by direct RIA FT was measured by RIA	170 Caucasian females with RMPs aged 18–42 years (mean 32) normal BMI: 20–24.9 kg/m^2	8-10 am	At mid FP (days 5 to 8), the ovulatory (day 13 to 15), and mid LP (days 19 to 22) of MC	Both TT and FT changed significantly through the MC with a peak during ovulatory phase
Massafra *et al.* 2000[78]	TT, FT, A were measured by RIA	12 healthy eumenorrheic females	8-9 am	On days -12, -10, -8, -6, -4, -2, 0 (from mid cycle), 2, 4, 6, 8, 10, 12, and 14	T, FT and A were highest during late luteal phase.
Mathor *et al.* 1985[81]	TT was measured by RIA. FT was measured by EqD	24-40 years, normal weight 10 females with ovulatory regular menstrual cycles	Not available	Mid cycle was considered Day 0 and BSC at 4 points in MC; from -9 to -7 (early FP), from -3 to -1 (late FP), from +3 to +7 (early LP), from +8 to +14 (late LP)	TT significantly lower in LP, fell progressively during the MC, whereas percent FT increased from the FP to the LP. FT fell significantly in late LP
Bancroft *et al.* 1991[89]	TT, FT, SHBG (method not reported)	53 females not using oral contraceptives (mean age 21.6 years, SD 3.2)	at noon and 2pm	Four points in the MC, at weekly intervals starting from day 1	There were no significant differences in plasma levels of T across phases

TT- total testosterone, **FT**- free testosterone, **A**- androstenedione, **DHT**- dihydrotestosterone, **DHEAS**- dehydroepiandrosterone sulphate, **RIA**- radio immunoassay, **MC**- menstrual cycle, **FP**- follicular phase, **LP**- luteal phase, **EFP**- early follicular phase, **MFP**- mid follicular phase, **MLP**- mid luteal phase, **SHBG**- Sex hormone binding globulin, **FAI**- Free androgen index, **EqD**- equilibrium dialysis, **ELISA**- enzyme-linked immunosorbent assay, **LC-MS/MS**- liquid chromatography with tandem mass spectrometry, **BSC**- blood samples collected, **RMPs**- regular menstrual periods, **ECLIA**- electrochemiluminescence immunoassay

Characteristics of studies

The included studies were conducted between years 1972 and 2020 and the number of participants ranged from 6 to 259. The studies concerned different ethnicities from North and South America, Europe, the Middle East, Asia and Australia. The females included were aged 18-45 years with a BMI ranging from 18 to 35 kg/m^2. The T assessment methods varied between studies.

Timing in the menstrual cycle

Testosterone measurements were done at variable time points in the MC and few studies reported daily or every other day (EOD) serum sampling[36-39]. Some studies assessed T at four points in the MC i.e., early and late in the follicular phase (FP) and early and late in the luteal phase (LP) [40-42]; some at three points, i.e., early FP, mid cycle (close to ovulation) and LP [43-45]; and others at two points, i.e., in middle of the FP and the LP respectively [46-48].

Multiple studies report that serum TT and FT levels peak during mid cycle at ovulation [36-39, 45, 49-75]. However, T level variation during the rest of the cycle does not seem to show a consistent pattern. Eight studies reported higher values during the FP[41, 47, 76-81], while nine studies reported higher values during the LP[40, 44, 46, 48, 82-86]. (Table 3). Many studies reported no change in T across the MC[42, 43, 87-102]. Out of the eighteen studies which did not observe any cyclical change, eight had assayed only two

points (namely FP and LP) [89, 90, 92-95, 97, 98] and only four studies had sampled daily or EOD [87, 88, 96, 97].

Serum testosterone assessment technique

The included studies reported multiple methods of detecting serum T (Table 4). Studies which compared T detection methods, used more than one measurement method for T detection. Few studies assessed both TT and FT [37, 41, 42, 44, 47, 57, 68, 71, 78, 89], while the majority tested only TT [40, 43, 53, 61, 65, 70, 76, 79, 80, 83, 86, 91, 93].

Discussion

Testosterone peak at mid cycle

Most studies reported at least a minute mid-cycle increase in TT, which coincided with ovulation. Irrespective of the sample size and detection method, TT peaked during mid cycle in the studies where daily sampling was done. Studies which sampled T at two points during the MC did not observe a T peak as this could not be demonstrated by plotting only two measurements. Levels of T were lower in late LP and in the peri menstrual phase [61] and lowest close to menstruation consistently. Daily sampling is more accurate as the increased number of samples overcomes the diurnal variation and sampling bias.

The mid-cycle T increase is due to synthesis of T by the ovarian theca cells, mainly regulated by LH [10]. LH receptors are located on the theca cells during all stages of the MC. LH principally stimulates androstenedione production and, to a lesser degree, production of T in theca cells. Androstenedione is then transported to the granulosa cells where it is aromatized to estrone and then converted to estradiol by 17-β-hydroxysteroid dehydrogenase. In contrast, in the granulosa cells LH receptors only appear in mature follicles greater than 10 mm in diameter, i.e., in the antral follicle, which is most likely to develop as the dominant follicle[41, 103]. Ovarian androgen production and its conversion to estradiol are essential for follicle recruitment and the process of ovulation. Insufficient androgen production in the FP [14] and high androgen levels can lead to anovulation[104, 105]. Testosterone was significantly higher during the FP in females with anovulatory menstrual cycles [55]. Ovulation is triggered by the LH surge and it is plausible that a LH-mediated increase in ovarian androgen production occurs. The peak in T is unlikely to be due to adrenal androgen production or peripheral aromatization [69]. The mid-cycle androgen rise may accelerate follicular atresia and ensure that a single dominant follicle reaches the point of ovulation[68]. It is likely that androgens facilitate apoptosis and consequent follicle atresia only in mature antral follicles [104]. The mid-cycle increase in sexual activity could be attributed to the mid-cycle androgen spike[106, 107]. If high T is related to the LH surge at ovulation it must gradually recede from the FP to the LP and reported measurements showed both TT and FT to follow this pattern until the next FP [50, 60]. A reduction in SHBG is also reported in the mid cycle, coinciding with ovulation [45, 50] and possibly further increasing the FT levels, followed by an increase in SHBG levels during the LP [99].

The T rise in the mid cycle could affect smooth muscle and may assist ovulation and subsequent actions on the fallopian tube. The mechanism of action of T on smooth muscle and the inflammatory cascade needs to be studied further to understand the physiological basis for the rise in T during the MC.

The mid-cycle rise in FT was more prominent in younger females while it was not evident in older females aged between 43 and 47 years, who were cycling regularly and had normal levels of prolactin and thyroid-stimulating hormone [68]. The decreased concentrations of FT and androstenedione, without significant changes in SHBG, suggest that in older women these hormones are produced in lower quantities during the mid-cycle. It was concluded that this process was ovary dependent and not adrenal dependent as the observed changes varied with the stage of the MC.

Table 3 Summary of changes in testosterone during different menstrual cycle phases.

SCENARIO	NUMBER OF STUDIES DESCRIBING THE SCENARIO	REFERENCES OF STUDIES
No variation in T levels throughout the cycle	18	[42, 43, 87-102]
T level is higher during the follicular phase	8	[41, 47, 76-81]
TT and FT peak at the time of ovulation.	32	[36-39, 45, 49-75]
T is higher during the luteal phase	9	[40, 44, 46, 48, 82-86]
Total	67	

T- testosterone, **TT**- total testosterone, **FT**- free testosterone

Table 4 Types of testosterone assessment methods used in the selected studies.

ASSESSMENT METHOD	NUMBER OF STUDIES	
	ASSESSMENT OF TT	ASSESSMENT OF FT
Radio immunoassay	41	7
Enzyme-linked immunosorbent assay	3	1
Chemiluminescent immunoassay	2	
Liquid chromatography tandem mass spectrometry	8	
Gas chromatography mass spectrometry	2	
High-performance liquid chromatography	1	
Enzyme-linked fluorescent assay	2	
Immunoradiometric assay	2	1
Equilibrium dialysis		3
Electro chemiluminometric assay	2	1
Chemiluminescent microparticle immunoassay	1	
Calculation	1	3

Variation in reported mid-cycle testosterone values

Mid-cycle FT has been reported to be 63ng/dL (23-120ng/dL) [50] and as low as 23.72 ± 15.35 ng/dL [52]. However, most of the values reported in the included studies were within the currently accepted reference ranges (0.5-2.5 nmol/L or 15-75 ng/dL) [20]. The variability of reported serum FT values was dependent on several factors such as study design, study sample (age and BMI), assay techniques and their limitations, low T threshold and its statistical interpretation. The studies incorporated in the review mainly concerned apparently healthy females. Obesity, hypothyroidism, acromegaly, and diabetes mellitus, as well as glucocorticoids and anabolic steroids are known to decrease SHBG, resulting in low total T and high FT levels; SHBG levels are also known to decrease with old age, liver disease, and anticonvulsant and estrogen use, resulting in altered T profiles [18]. Higher levels of TT and FT with low SHBG have been reported in females with polycystic ovarian syndrome (PCOS) [108]. Studies involving women with chronic medical conditions, metabolic comorbidities like PCOS, diabetes mellitus, and other endocrine abnormalities are not within the scope of this review, and were therefore not included. However, BMI was seen to vary with different studies, with some studies also including females with higher BMI values. Of the limited number of studies which also evaluated overweight and obese females, some studies reported low TT [86], while others reported consistently high TT and FT values throughout the MC [41].

Variation of SHBG

An increase in SHBG in the LP compared with the FP was reported for ovulatory MCs [47, 54, 98, 109]. However, no consistent pattern of changes in SHBG, TT or FT levels was observed. Only a few studies had a sample size of more than 20 [42, 44-51, 77, 83], which could also affect these reported outcomes.

Timing of collection of samples during the menstrual cycle

As regards the definitions of the phases of the MC and the timing of sample collections, the studies showed considerable differences. When the FP was considered to last from day 0 to ovulation and the LP from ovulation to beginning of next menstrual cycle, there was uncertainty over the best day to sample in each of these two phases. Some studies, however, considered a range of days after the beginning of the MC for drawing blood in the FP, while mid cycle was defined as day 14-15 and the LP as after the mid cycle. The mid cycle was considered the late FP in some studies. In most studies at least three samples were taken, one during the FP, one at mid cycle, and one during the LP [43-45]. Instead, the few studies analyzing daily blood samples did not encounter the problem of determining ovulation and provided the best evidence for changing patterns of T [39, 62, 63, 100].

The selection of females

Most studies included females of reproductive age with regular menstrual cycles and they ranged from adolescents to premenopausal females. The participants also varied in terms of marital status and parity.

Although changes in T depend on whether a cycle is ovulatory or anovulatory, most of the studies did not identify MCs as ovulatory or anovulatory. Studies that did identify ovulatory and anovulatory cycles reported different patterns of T fluctuation, where in anovulatory cycles no mid-cycle rise in T was seen, with one study reporting higher T during the FP [56]. In contrast, in some studies no difference in T were seen between the females with ovulatory and anovulatory cycles [67].

BMI, smoking, alcohol consumption and exercise are known confounders for T levels in females [24]. However, very few studies have controlled for such confounding factors. Non-smoking females, without any chronic medical disease, who were not on any medication were studied in well planned studies omitting the errors due to the above factors [52, 78].

Sample timing during the day

It is important that timing of the sampling is uniform at all points of the MC as hormone levels are known to decrease after waking due to diurnal variation [110, 111]. In most studies sampling was done in the morning to get the highest T levels, and after an overnight fast. Fasting overnight was important as food interferes with the results of the analysis [112]. Some studies did sampling throughout the day and these provide the best evidence for the highest peak of T during the day.

Method of assessing testosterone

Most studies used the RIA method to measure TT, while some used the gold standard LC-MS/MS. Others used ELISA, CLIA, HPLC, ELFA. Older studies used RIA with organic extraction and chromatographic separation to remove interfering substances and matrix effects.[20] RIA has sensitivity to detect higher concentrations of hormone while errors may occur at lower concentrations especially in the late LP when hormone concentrations are lower. Recent studies have used automated platforms with nonradioactive methods like chemiluminescent detection. Liquid chromatography mass spectrometry (LC-MS) and LC-MS/MS can assess low concentrations of T seen in the MC [20].

It was difficult to compare studies as the assay techniques used and the T fraction tested (TT or FT) differed between studies; furthermore, outcomes were expressed in different forms, such as the T ratio or the FT index (calculated from TT and SHBG).

Future research on T changes during the MC needs to be designed to address the current knowledge gaps. MC phases and timing of blood sampling should be well defined. Future study protocols should include at least three sampling points per MC as a peak cannot be demonstrated when plotting only two measurements. Daily sampling, however, will overcome problems encountered in defining the phases of the MC and in detecting ovulation for mid-cycle sampling. The use of daily, morning samples, taken from healthy regularly menstruating females and tested using the liquid chromatography method, is recommended as the best option to achieve coherent results.

Conclusions

Both TT and FT peak at ovulation in females with regular (ovulatory) menstrual cycles. In the early LP, TT levels appear to be higher than in the early FP, but levels during the late LP

seem to be lower than in the mid-follicular phase. Variations in serum T levels during different phases of the menstrual cycle seem to be related to whether the MC is ovulatory or anovulatory, to the T assessment technique used, and the timing of sample collection both during the MC and during the day. Further research should be conducted on larger populations and using more clearly defined criteria.

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