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## Measuring testosterone: the power of a method on steroids

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## dynamics of serum testosterone during the menstrual cycle evaluated by daily measurements with an ID-LC-MS/MS method and a 2<sup>nd</sup> generation automated immunoassay

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## ABSTRACT

Testosterone concentrations in normally cycling women are assumed to be elevated around the time of ovulation. The clinical relevance of changing testosterone concentrations during the menstrual cycle, however, is unclear. Poor performance of current direct immunoassays for testosterone at low concentrations confounds this issue. Therefore, our objective was to assess daily testosterone fluctuation during the menstrual cycle by a thoroughly validated isotope dilution-liquid chromatography-tandem mass spectrometry (ID-LC-MS/MS) method and to evaluate whether an ARCHITECT® 2<sup>nd</sup> Generation Testosterone fully automated immunoassay is equally suited for this purpose.

**METHOD.** Testosterone was measured in serum obtained daily during the menstrual cycle of 25 healthy women, characterized by biochemical and physical examination.

**RESULTS.** Performance of the ID-LC-MS/MS method was concordant with a published reference method ( $y = 1.007x - 0.056$  nmol/L;  $r = 0.9998$ ). Comparison of the immunoassay to ID-LC-MS/MS yielded  $y = 1.095x + 0.104$  nmol/L ( $r = 0.9031$ ). Overall, testosterone concentrations were higher mid-cycle, but a peak was not discernible in each individual. Apart from a persistent positive bias, the immunoassay measured the same testosterone profiles as the ID-LC-MS/MS method. The reference interval in women was 0.30 – 1.69 nmol/L (8.7 – 48.7 ng/dL) for ID-LC-MS/MS and 0.50 – 2.00 nmol/L (14.4 – 57.7 ng/dL) for the immunoassay.

**CONCLUSION.** The elevation of mid-cycle testosterone concentrations is statistically significant, although not clinically relevant since day-to-day variation is higher and independent of the menstrual cycle. In this light, a single testosterone measurement might not be reflective of the overall testosterone status in an individual. Measurements obtained using the 2<sup>nd</sup> generation immunoassay gave comparable results across the menstrual cycle.

## INTRODUCTION

Although the physiological role of testosterone in women is still incompletely defined, various pathological states are associated with significantly altered testosterone concentrations. Currently, testosterone concentrations in women are important for identifying androgen secreting tumors and for evaluating gonadal and adrenal functional disturbances such as hypopituitarism, adrenal insufficiency, polycystic ovary syndrome, and hirsutism. Accurate, method specific testosterone reference ranges are needed to differentiate between the normal physiological and pathological state. Establishment of reliable reference ranges, however, is cumbersome. Not only is measuring low testosterone concentrations typically found in women analytically challenging [1], testosterone concentrations also undergo subtle changes under

physiological influences such as diurnal variation [2], menstrual cyclic variation, and reproductive maturity (pre- and post-menopause) [3]. Particularly menstrual cycle-related changes have been studied for their impact on the determination of reference ranges and in order to formulate recommendations regarding the timing of sampling.

Previous reports on cyclic variation and reference ranges in women suffer from several limitations. First, direct immunoassays often used to determine serum total testosterone in women are notorious for their poor performance at low testosterone concentrations which are found in women [4;5]. Overestimation of testosterone due to cross-reaction with other steroids is one of the important causes for this suboptimal performance. Second, specifically with regard to cyclic variation, most studies are limited by the use of samples taken at only 3 time points per menstrual cycle, per woman [6;7]. A few reports describe reference intervals established by LC-MS/MS, however cyclic variation was not taken into account or evaluated [8-10].

Because of the limitations of previously reported studies, the question remains whether menstrual cycle-related changes are relevant in clinical practice and cycle-related reference ranges have to be determined. To answer this question we performed this study in which testosterone was determined by isotope dilution-liquid chromatography-tandem mass spectrometry (ID-LC-MS/MS) in serum samples drawn daily during a menstrual cycle from healthy, well-characterized women. In this paper we also report on the comparison of this ID-LC-MS/MS testosterone method and the reference method by Thienpont *et al.* [11]. Moreover, since ID-LC-MS/MS is not accessible to all clinical laboratories, we compared the results to those obtained using a next generation direct immunoassay with improved performance and accuracy.

## EXPERIMENTAL SECTION

### EXPERIMENT I: COMPARISON OF ID-LC-MS/MS METHOD TO REFERENCE METHOD

Sample preparation was performed according to a method described previously [3]. In short, a calibration curve was prepared with each batch and all specimens were run in duplicate. A stable isotopically labeled internal standard (testosterone-2,2,4,6,6-D<sub>5</sub>; D<sub>5</sub>T; CDN Isotopes, Pointe-Claire, Canada) was added to every specimen (sample, control, calibrator) prior to work up. Testosterone was extracted with hexane/ether (4/1, v/v), dried, and derivatized with methoxylamine hydrochloride (T-mox), followed by another hexane/ether extraction. Separation was achieved on a C<sub>8</sub> analytical column (XBridge, 2.1 x 50 mm, 2.5 μm particle size: Waters Corp., Milford, MA) by gradient elution, using 0.1% formic acid in water and 0.1% formic acid in acetonitrile. A Quattro Premier XE tandem mass spectrometer (Waters Corp.) with electrospray in positive mode was used for detection. For each component (analyte and internal

standard), two transitions were monitored:  $m/z$  318 > 138 and  $m/z$  318 > 126 for T-mox;  $m/z$  323 > 142 and  $m/z$  323 > 129 for D<sub>5</sub>T-mox. The first transitions in each set were used for quantification, the second transitions for confirmation. Data acquisition and processing were done with Masslynx 4.1 software (Waters Corp.). The ID-LC-MS/MS method features a limit of quantification of 0.10 nmol/L (2.88 ng/dL); inter-assay variation at 0.21, 2.1, and 15.8 nmol/L was 9%, 7%, and 4%, respectively. The ID-LC-MS/MS method was compared to a certified reference method isotope dilution-gas chromatography-mass spectrometry (ID-GC-MS) [11] by measuring, in three independent runs, a set of 40 serum samples drawn from males as well as females ranging from 0.20 to 31.3 nmol/L (5.8 – 902.8 ng/dL) as assigned by ID-GC-MS. Details of the ID-GC-MS method have been described previously by Thienpont *et al.* [11].

#### EXPERIMENT 2: TESTOSTERONE CONCENTRATIONS ACROSS THE MENSTRUAL CYCLE

*Subjects.* The study protocol was reviewed and approved by an institutional review board. Healthy women were recruited at the Massachusetts General Hospital (Boston, MA). All subjects recruited provided written informed consent. Subjects were questioned for use of medication, including over the counter steroids such as DHEA. The included subjects did not use medication other than multi-vitamins, allergy and/or over the counter pain reducing medicine (i.e., Advil, Aleve, ibuprofen, Tylenol, Motrin, Midol, Claritin). Subjects had to have at least 2 previous regular menstrual cycles prior to admittance into the study.

Physical examination was performed on 36 women. In total 9 women were excluded due to hirsutism (4 subjects excluded), acanthosis nigricans (3 subjects excluded), nodules present at head/eyes/ears/nose/throat exam (1 subject excluded), and heart murmurs (1 subject excluded). The results of the physical examination were normal for 27 women and included, beside the above mentioned: normal ultrasound of the ovaries, normal thyroid size and consistency, breasts without masses, chest clear to auscultation, normal heart sounds, soft and non/tender abdomen: no masses or visceromegaly, normal extremities, central and peripheral nervous system normal.

After physical examination, the study included daily morning serum samples of 27 women, in total 15 samples were missing (max 2 missing specimens per subject). Biochemical examination was performed and included measurement of serum luteinizing hormone (LH), progesterone and testosterone. An LH peak > 9.1 IU/L, which was set as day 0, was observed and ovulation was confirmed by progesterone > 5.1 nmol/L (> 1.6 ng/mL) during the luteal phase and by ultrasonography in 25 out of 27 women.

For the statistical analysis of testosterone concentrations across the menstrual cycle, samples from two women were excluded because their cycles were non-ovulatory as judged by the absence of elevated progesterone concentrations during luteal phase.

Furthermore, due to insufficient volume, testosterone was not determined in 3 serum samples. Seven specimen with low concentrations ( $< 0.45$  nmol/L) yielded discordant results when assayed undiluted by the new generation immunoassay (see Experiment 3) as prescribed in the manufacturer's instructions ( $> 0.6$  nmol/L) and were, therefore, excluded from statistical analysis.

The 25 healthy women included in the statistical analysis of testosterone concentrations across the menstrual cycle were 27.3 (19 – 39) years of age (mean; range) and had a BMI of 23.3 (18 – 29) kg/m<sup>2</sup>. Duration of menstrual cycle was 28 (22 – 32) days. The cohort consisted of 60% white, 20% Hispanic, 8% black, 12% other women; 76% non-smokers, 12% ex-smokers and 12% smokers; 28% non-alcohol drinkers, 68% light-alcohol drinkers, and 4% moderate-drinkers.

*Samples.* Blood samples were drawn daily in the morning between the first day of menses and the first day of next menses. Blood was drawn into 10 mL glass vacutainers [no preservative] and allowed to clot at 2 – 8 °C for 1 – 12 h. Serum was separated from clots by centrifugation [3,000 – 3,200 g for 10 minutes], aliquotted into polypropylene tubes and stored capped at -25 to -35 °C. Aliquots were shipped on dry ice for measurement of testosterone.

*Assay.* Testosterone was measured by ID-LC-MS/MS which was performed at the VU University Medical Center as described under Experiment 1. In addition, LH and progesterone were determined immunochemically by AxSYM (Abbott Diagnostics) at the Massachusetts General Hospital. All samples from the same individual were analyzed within a run to minimize intra-individual variation and this workflow was also utilized for the ID-LC-MS/MS analyses.

#### EXPERIMENT 3: NEW GENERATION TESTOSTERONE DIRECT IMMUNOASSAY

Testosterone in all specimens from Experiment 2, ovulatory and non-ovulatory cycling women ( $n = 27$ ), was also determined by an automated delayed one step chemiluminescent microparticle immunoassay, the ARCHITECT® 2nd Generation Testosterone (Architect II) (Architect i2000 analyzer; Abbott Diagnostics), that has recently become available. First, 50  $\mu$ L sample is automatically diluted with 100  $\mu$ L specimen diluent (phosphate buffered saline with preservative). Then assay specific diluent and ovine monoclonal antibody coated magnetizable particles are added. After incubation testosterone-acridinium tracer is added, followed by washing, addition of pretrigger and trigger solutions, and the light output is measured. The testosterone concentration is interpolated from a calibration curve derived from 6 calibrators (0 – 30 nmol/L; 0 – 865 ng/dL). Options to measure very low concentrations, i.e. below 0.6 nmol/L (13.0 ng/dL), undiluted and high concentrations automatically diluted 1 in 4 (instead of the standard 1:2 dilution) are offered on the instrument. The limit of quantification of the assay (including undiluted measurement option) was 0.1 nmol/L (2.88 ng/dL). The measurements were performed within the same lot of reagent.

Intra-assay and inter-assay were assessed at the VU University Medical Center: intra-assay variation at 0.2, 2, and 20 nmol/L were found to be 8%, 4%, and 4% ( $n > 20$ ), respectively, and inter-assay variation at the same concentrations were found to be 10%, 6%, and 6% ( $n > 20$ ), respectively.

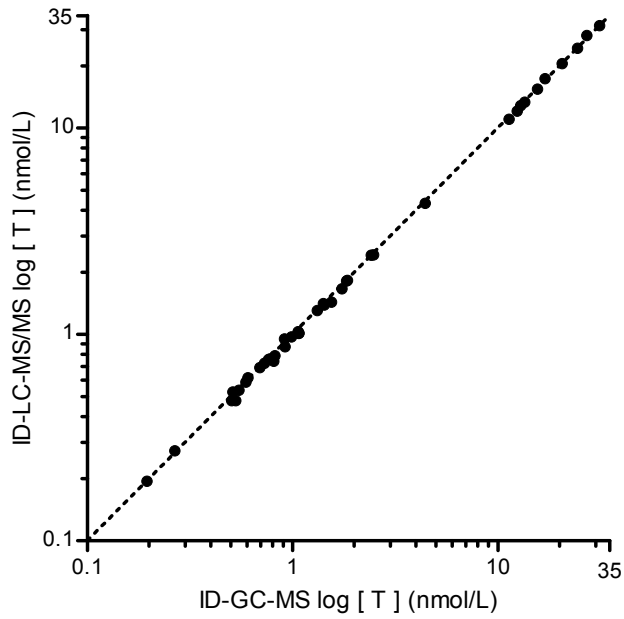
#### DATA ANALYSIS

Method comparison was done by weighted Deming regression analysis and Pearson correlation using Analyse-It software (Analyse-It Software, Ltd., Leeds, United Kingdom). Days of the menstrual cycle with  $n > 10$  values (day -12 to 16) were used in the statistical analysis, which was performed using SPSS software (version 15.0). To minimize skew, testosterone concentrations were log transformed after adding a constant to the original concentrations in order to obtain an optimal transformation. The constant for each variable was computed by the procedure `lnskew0` of Stata 11.2 software (StataCorp, Texas, USA). To account for dependency of the data due to repeated measurements within women, a compound symmetric covariance structure was chosen. Pair wise comparison between the reference day (day 0) and all other days was executed as follows: testosterone concentrations of all 25 women were aligned on the day of LH peak and this day was set as the reference day (day 0). The mean concentration of 25 women on each day were calculated and compared pair wise to the mean concentration on the reference day. In this case, means of day -12 to 16 were each compared to the mean on day 0, which resulted in 28 comparisons. Adjustment for multiple comparison was done by applying Bonferroni correction. P values  $< 0.05$  were considered to reflect statistical significance. Values are reported as mean (95% confidence interval) unless indicated otherwise.

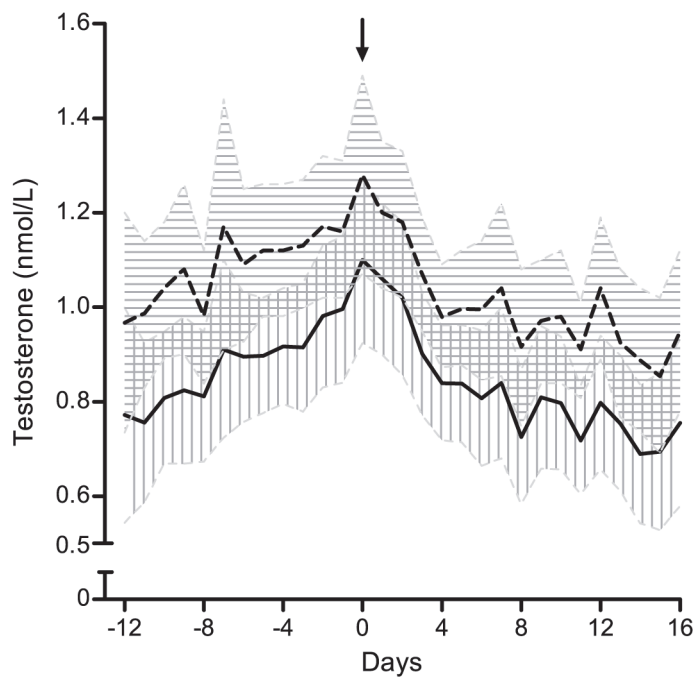
## RESULTS

### *Experiment 1: Comparison of ID-LC-MS/MS method to the reference method*

The ID-LC-MS/MS was validated by measuring 40 serum samples in three independent runs with testosterone concentrations between 0.20 and 31.3 nmol/L (5.7 – 902.8 ng/dL) as determined by a reference method (ID-GC-MS). Comparison between ID-GC-MS and ID-LC-MS/MS by weighted Deming regression analysis showed a slope of 1.007 (0.990 – 1.023), intercept of -0.056 nmol/L (-0.100 to -0.012), and Pearson  $r$  of 0.9998 (0.9996 – 0.9999) as shown in figure 1.



**FIGURE 1:** Weighted Deming regression analysis of mean testosterone concentrations measured by ID-GC-MS and ID-LC-MS/MS:  $y = 1.007x - 0.056$  nmol/L ( $r = 0.9998$ ;  $n = 40$ ). Dotted line represents the line of identity ( $x = y$ ). Note the logarithmic scales. To convert testosterone concentrations to ng/mL, multiply by 0.3.



**FIGURE 2:** Daily mean testosterone concentrations. Solid line: concentrations by ID-LC-MS/MS with the 95% confidence interval (vertical pattern). Dashed line: concentrations by Architect II immunoassay with the 95% confidence interval (horizontal pattern). The arrow indicates day 0 (day of LH peak value).

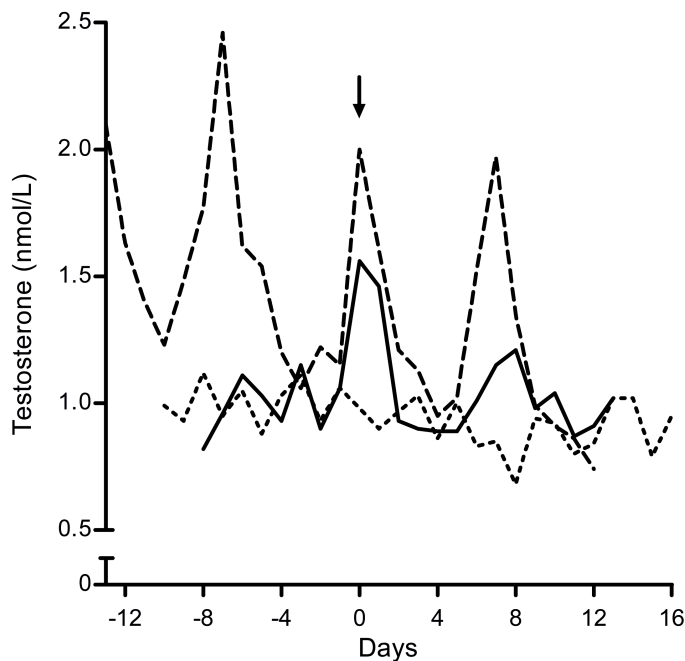
**TABLE 1:** Days during the menstrual cycle on which mean testosterone concentrations were found to be significantly lower ( $p < 0.05$ ) than the mean concentration on day 0.

	Follicular phase	Luteal phase
Testosterone (ID-LC-MS/MS)	-12 to -3	3 to 16
Testosterone (immunoassay)	-12 to -10, -8, and -6	3 to 16



### Experiment 2: Testosterone concentrations during menstrual cycle

Daily mean testosterone concentrations measured by ID-LC-MS/MS and immunoassay are shown in figure 2. At the group level a peak was seen on the day of LH peak (day 0) by ID-LC-MS/MS as well as by Architect II immunoassay. Statistically significant higher mean testosterone concentrations compared to the follicular and luteal phase were found as summarized in table 1. At the individual level, by contrast, not all women showed one distinct testosterone peak around ovulation. Figure 3 displays the testosterone profiles during the normal menstrual cycle of 3 different women: one individual showed 3 peaks, one woman showed a distinct peak around ovulation and a small peak during the luteal phase, and one individual showed no elevated testosterone concentrations during the cycle (see Supplemental figure 1 for all 25 individual cycles). The ratio of extremes (max/min) in each individual was calculated and mean ratio was 2.4 (range 1.6 – 3.7) by ID-LC-MS/MS and 2.1 (range 1.5 – 3.4) by immunoassay.

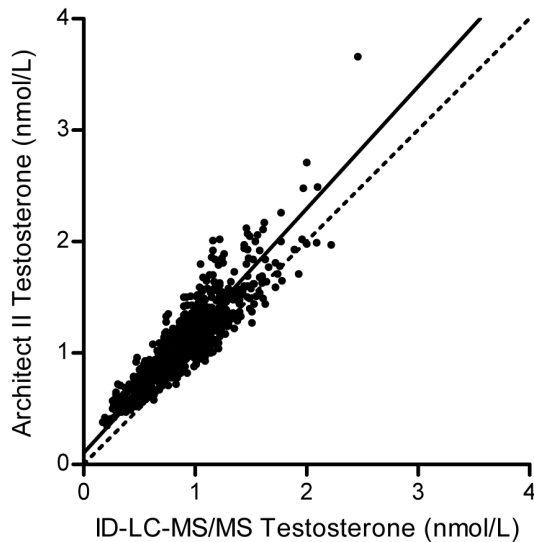


**FIGURE 3:** Testosterone profile, measured by ID-LC-MS/MS, during a normal ovulatory menstrual cycle in three individuals. The arrow indicates day 0 (day of LH peak value).

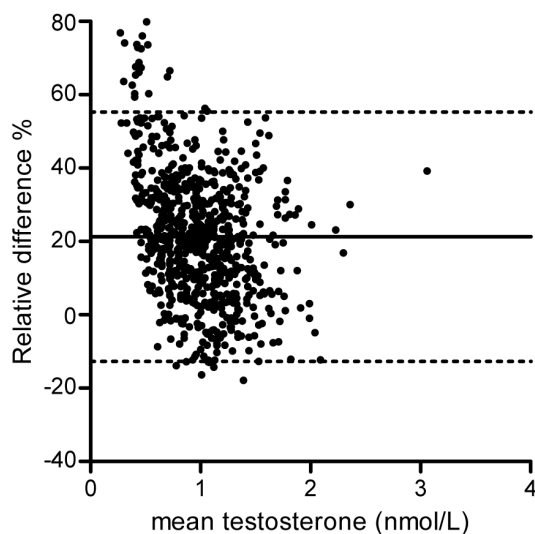
To determine the reference interval for testosterone, data obtained from 25 healthy women with an ovulatory menstrual cycle were analyzed using a compound symmetric covariance structure and the 95% prediction interval was calculated. For ID-LC-MS/MS, the reference interval for testosterone in healthy women thus was found to be 0.30 to 1.69 nmol/L (8.7 – 48.7 ng/dL). Similarly, the reference interval for testosterone by Architect II immunoassay was 0.50 to 2.00 nmol/L (14.4 – 57.7 ng/dL).

*Experiment 3: New generation testosterone direct immunoassay*

Testosterone was determined by ID-LC-MS/MS and Architect II immunoassay in serum samples of 27 women ( $n = 743$ ). Weighted Deming regression analysis showed a slope of 1.095 (1.032 – 1.157), intercept of 0.104 nmol/L (0.053 – 0.154), and Pearson  $r$  of 0.9031 (0.8889 – 0.9156) (figure 4). Figure 5 illustrates the relative difference between ID-LC-MS/MS and Architect II. When the testosterone concentrations by ID-LC-MS/MS and immunoassay were compared per menstrual phase, an overall mean bias of the immunoassay of 23%, 17%, and 24% in the follicular phase (day -12 to -4;  $n = 186$ ), ovulatory phase (day -3 to 3;  $n = 170$ ), and luteal phase (day 4 to 16;  $n = 279$ ), respectively, was observed. The profiles of the differences between ID-LC-MS/MS and immunoassay per individual menstrual cycle were diverse and the mean difference per woman ranged from 1% to 47%. Supplemental figure 2 illustrates the overall bias of all 25 woman across the menstrual cycle.



**FIGURE 4:** Weighted Deming regression analysis of testosterone concentrations measured by ID-LC-MS/MS and Architect II. Solid line represents weighted Deming regression:  $y = 1.095x + 0.104$  nmol/L ( $r = 0.9031$ ;  $n = 743$ ), dotted line is the line of identity ( $x = y$ ).



**FIGURE 5:** Bland-Altman plot: the average testosterone concentration of ID-LC-MS/MS and Architect II ( $n = 743$ ) is plotted on the x-axis and the y-axis represents the relative difference ( $((\text{Architect II} - \text{ID-LC-MS/MS}) / \text{average}) \times 100$ ). Solid line represents the mean difference (21.3%), dotted lines are the 95% confidence interval (-12.7 to 55.3%).

## DISCUSSION

This study included 25 healthy normal cycling females who were carefully screened by biochemical and physical examination. Total serum testosterone was assessed daily across a menstrual cycle, using a thoroughly validated ID-LC-MS/MS assay, to evaluate cycle-related changes in testosterone and determine clinically useful reference ranges. Furthermore, results were compared to those obtained using a new generation automated direct immunoassay. This is important because while LC-MS/MS methods are widely available in reference laboratories they are expensive and not widely available to many clinical laboratories.

The dynamics of testosterone across the menstrual cycle has been addressed in literature. In general, elevated testosterone concentrations were found during the ovulatory phase. However, the majority of these studies were limited to three samplings and testosterone measurements were done using immunoassay, which are notorious for their poor performance at concentrations generally found in women. Among the more conclusive studies, Goebelsmann *et al.* [12] and Braunstein *et al.* [13] used more samplings and an immunoassay in combination with extraction and chromatography which is superior to direct immunometric methods. They also found elevated testosterone concentrations during the ovulatory phase. However, Braunstein *et al.* pointed out that the subjects in the study were self-proclaimed healthy and samples were not drawn daily, whereas Goebelsmann *et al.* tested a small number of subjects. In our study, we have tried to be conclusive by using an adequate ID-LC-MS/MS to study the *daily* dynamics of testosterone during the menstrual cycle in a substantial number of carefully screened subjects. Our finding that, at the group level, serum testosterone is statistically significant elevated in the ovulatory phase confirms previous studies.

By sampling daily during the menstrual cycle and analyzing the data of each woman individually, elevated testosterone concentrations mid-cycle were not discernible in every woman, as shown in figure 3 and in the Supplemental data. Intra-individual variation surpasses the group average in the menstrual cycle: consecutive measurements differ substantially, the ratio of extremes (max/min) found in each woman ranged from 1.6 to 3.7. Based on literature, we speculate that this might be due to the pulsatile secretion of testosterone which exceeds circadian and cycle rhythm [2]. Recommendations regarding optimal time of sampling for testosterone in women are contradictory [7;14]. According to our data, we conclude that sampling timing, i.e. day of the menstrual cycle, is not critical with respect to reference ranges that can be applied irrespective of the menstrual cycle. However, because daily testosterone concentrations in an individual woman can differ as much as 3.7-fold, a single testosterone measurement for diagnostic purposes might not be reflective of the

overall testosterone status in an individual. It is important to re-emphasize the basic principle that interpretation of testosterone concentrations should always be done relative to method-specific reference interval and in the context of the clinical setting.

In this study, the reference range for serum testosterone in healthy premenopausal women by a thoroughly validated ID-LC-MS/MS was determined to be 0.30 to 1.69 nmol/L (8.7 – 48.7 ng/dL). Other studies reporting on reference ranges in healthy women by ID-LC-MS/MS did not take cyclic variation into account, however, in our study the application of serum testosterone reference range is valid for all phases of the menstrual cycle and the reference ranges in other studies show close agreement with the reference range in this study. The subtle differences might not be due to the ID-LC-MS/MS method used, as all methods, including in this study, were thoroughly validated and calibrated against a certified gas chromatography-mass spectrometry reference method [11]. A possible explanation may be the differences in study population: Barth *et al.* [8] specified non-PCOS values and not reference values (< 1.8 nmol/L), Kushnir *et al.* [9] used apparently healthy volunteers (reference range 0.31 – 1.91 nmol/L), and Haring *et al.* [10] did not evaluate the presence of polycystic ovarian syndrome (PCOS) in their patients and thus not used as an exclusion criterium (0.46 – 1.95 nmol/L).

Multiple factors, both physiologically and external, contribute to circulating testosterone. In this study, we have tried to take these factors into account by carefully screening the included healthy women and drawing daily samples during a menstrual cycle. However, the subjects in this studied group covered a relatively small age-range [10] and our clinical screening did not include diet [3], exercise [15], depression, and sexual disturbances [7] which may alter testosterone concentrations.

The prevailing dogma suggests that approximately half of the circulating testosterone comes from the ovary (which contributes largely to the dynamics of the menstrual cycle) and half from the adrenal cortex which is relatively constant. Since there is growing evidence that adrenal androgen production is less constant than previously assumed and varies with age, ovarian stage, and ethnicity, then there is interest if these differences can be discerned from the measure to total testosterone. Such an analysis would have bearing on not only the higher concentrations, but on the ability to observe pulsatility particularly at the lower range of values. To assess the relative importance of fluctuations in the contribution of the adrenal glands to the concentration of testosterone in the peripheral circulation is beyond the scope of this manuscript. A proper experimental set-up to arrive at a conclusive answer to this question might require adrenal and ovarian sampling over a long period of time and assessment of concentrations of steroids of adrenal and ovarian origin in these specimens as well as in peripheral samples. It is considered highly unlikely that ethical approval for such a study could be obtained.

The analytical part of this study included the validation of our ID-LC-MS/MS method and a new generation immunoassay, ARCHITECT® 2nd Generation Testosterone. The ID-LC-MS/MS method had adequate sensitivity and specificity for measurement of testosterone in women and was well-correlated with a gold standard ID-GC-MS [11]. Liquid chromatography tandem-mass spectrometry (LC-MS/MS) has been proposed as the preferred method for measuring low concentrations of testosterone typically found in women and children [1]. Indeed, some LC-MS/MS methods show a good performance compared to the reference method ID-GC-MS [11]. However, discrepancies between different LC-MS/MS assays have also been reported [11;16]. This underlines the conclusion that thorough validation is also requisite for a technique such as LC-MS/MS [11] and until testosterone measurements are standardized method-specific reference intervals for LC/MS-MS methods must be established.

LC-MS/MS methods are not accessible to all clinical laboratories. Thus, sensitive and accurate high-throughput testosterone immunoassays are still needed. The Architect II automated immunoassay has been developed to improve accuracy and sensitivity of a direct method. Hence this assay was compared to ID-LC-MS/MS in the studies reported here. The immunoassay measurements resulted in the same testosterone profile across the menstrual cycle, but with a small positive bias. Based on the weighted Deming regression equation and the correlation coefficient, the performance of Architect II immunoassay for testosterone concentrations typically found in women is superior to the 10 immunoassays tested by Taieb *et al.* [4] and the other 6 immunoassays tested by Tiel Groenestege *et al.* [5]

The overall mean bias of the immunoassay compared to the ID-LC-MS/MS tends to be higher in the follicular and luteal phase compared to the ovulatory phase. This is in line with the lower bias of the immunoassay in the upper range of serum testosterone in females (figure 5) and the average higher testosterone concentrations observed in the peri-ovulatory period. However, the relatively low bias during the ovulatory phase that was seen on average could not be observed when analyzing the data per individual. This may be related to the profile of testosterone concentrations in individual women. The difference between ID-LC-MS/MS and immunoassay seems to be more dependent on the individual as the individual difference profiles across the menstrual cycle were diverse and the mean difference per woman ranged from 1% to 47%. Possible causes might lie in differences in androgen-profile and differences in binding protein-profile in individuals. If these profiles remain stable across the menstrual cycle, while testosterone concentration tends to fluctuate, the relative degree of cross-reaction in the immunoassay might be increased when testosterone is decreased in the same individual. Further investigation is needed to determine whether this phenomenon is due to analytical error, or has to be attributed to physiological variation in the serum steroid profile during the menstrual cycle.

When following the manufacturer's instructions, no testosterone concentration could be assigned to 7 out of 743 specimens: these samples yielded  $0.45 > \text{testosterone} > 0.6$  nmol/L. Looking at the ID-LC-MS/MS data for these specimens, we would recommend that the assay result is reported as  $< 0.45$  nmol/L if this is encountered in the future. Apart from this problem, the second generation testosterone immunoassay shows a positive bias compared with our LC-MS/MS which is in the same range as the bias that has been reported to exist between the results of some tandem-MS methods [16]. In that perspective, the performance of the new generation immunoassay is sufficient for clinical practice.

## CONCLUSION

Using an ID-LC-MS/MS method with adequate sensitivity and specificity to measure low testosterone concentrations at a group level, the elevated testosterone concentrations during ovulation are statistically significant. The clinical relevance, if any, still needs to be established, since the day-to-day variation in individuals is higher and independent of menstrual cycle-related changes. Given this day-to-day variance, a single testosterone concentration might not be reflective of the overall testosterone status. The Architect II immunoassay showed acceptable performance and concordance with LC-MS/MS to measure the relatively low testosterone concentrations seen across the menstrual cycle.

## ACKNOWLEDGEMENTS

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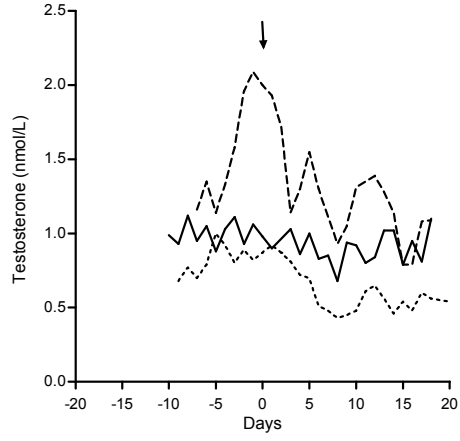
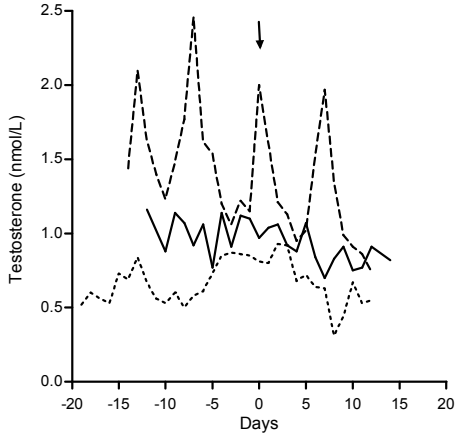
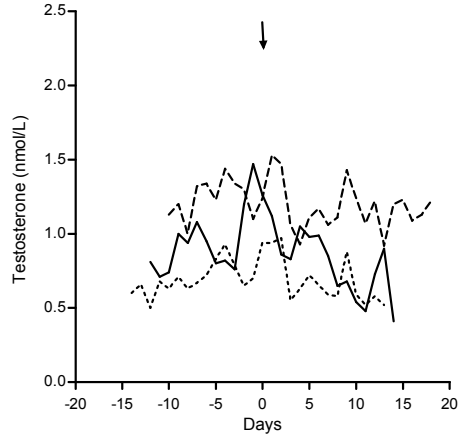
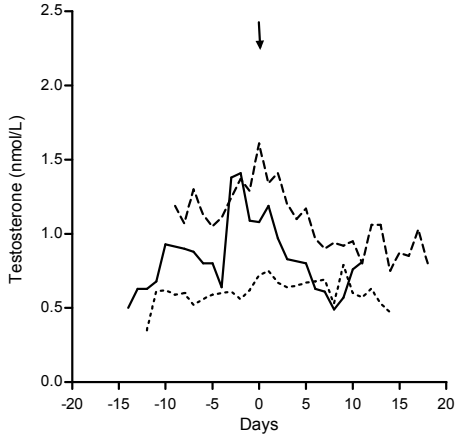
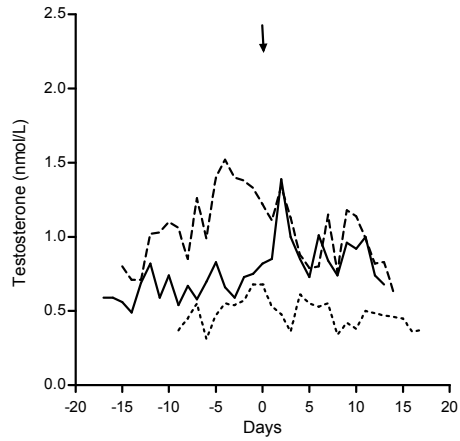
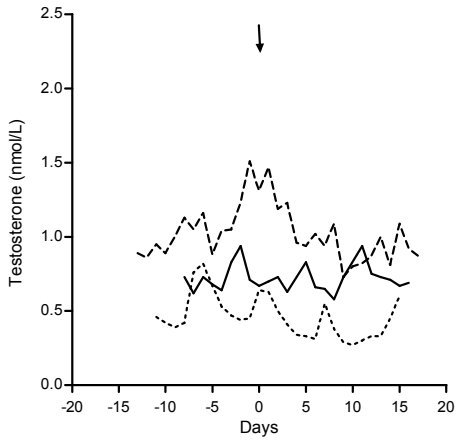
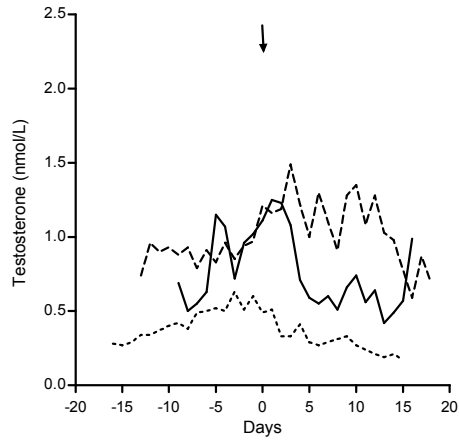
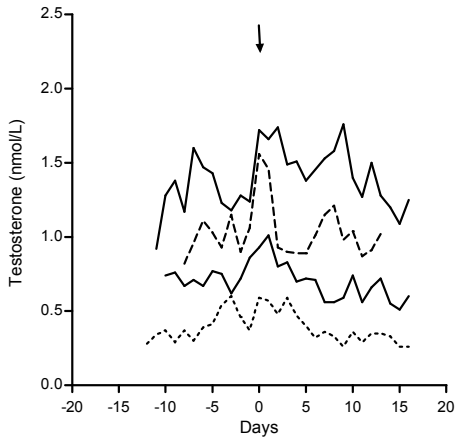
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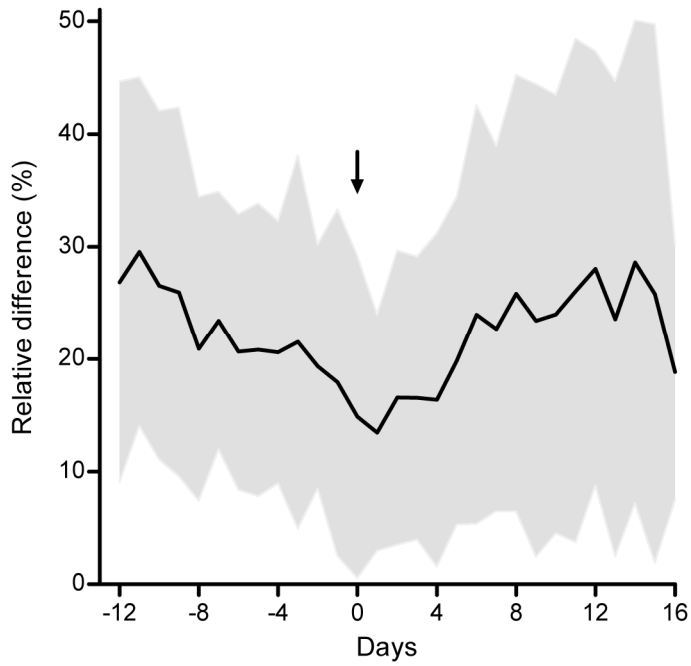
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## SUPPLEMENTAL FIGURES

**SUPPLEMENTAL FIGURE 1 (SEE NEXT PAGE):** Testosterone profiles, measured by ID-LC-MS/MS, during a normal ovulatory menstrual cycle of 25 individuals. The arrow indicates day 0 (day of LH peak value). The profile of 3 or 4 individuals are shown per figure. To convert testosterone concentrations to ng/mL, multiply by 0.3.







**SUPPLEMENTAL FIGURE 2:** The mean relative difference between testosterone concentrations measured by ID-LC-MS/MS and Architect II immunoassay in 25 healthy women across the menstrual cycle. The relative difference is calculated using the mean testosterone concentrations per day per method:  $(\text{immunoassay} - \text{ID-LC-MS/MS}) / (\text{mean of both methods})$ . Solid line: mean relative difference on each day. The grey area represents the 95% confidence interval. The arrow indicates day 0 (day of LH peak value).



