Measuring testosterone: the power of a method on steroids

Bui, H.N.

2013

document version
Publisher's PDF, also known as Version of record

5

testosterone, free testosterone, and free androgen index in women: reference intervals, biological variation, and diagnostic value in polycystic ovary syndrome

Hong N. Bui, Patrick M. Sluss, Stuart Blincko, Dirk L. Knol, Marinus A. Blankenstein, Annemieke C. Heijboer

Manuscript in preparation

ABSTRACT

INTRODUCTION. The introduction of more accurate methods for testosterone analysis, such as liquid chromatography-tandem mass spectrometry (LC-MS/MS) or next-generation immunoassays, may assist in reaching a consensus on the definition of hyperandrogenism, one of the diagnostic criteria of polycystic ovary syndrome (PCOS). The objective of our study was to determine reliable reference intervals and biologic variation for testosterone, free testosterone (fT), and free androgen index (FAI) in women and to test the discriminative value of these parameters in a PCOS-population.

METHODS. Serum was obtained daily during a normal menstrual cycle from 25 healthy women (677 data-points). A single serum sample was obtained from 43 PCOS-patients. Testosterone was measured by LC-MS/MS and by Architect® 2nd Generation Testosterone Immunoassay. Sex hormone-binding globulin was measured to calculate fT and FAI ($100 \times$ [T] / [SHBG]).

RESULTS. The reference intervals were calculated resulting in T = 0.3 – 1.6 nmol/L and 0.5 – 2.0 nmol/L, fT = 5.2 – 26 pmol/L and 7.2 – 33 pmol/L, and FAI = 0.4 – 2.9 and 0.6 – 4.4, by LC-MS/MS and immunoassay, respectively. The values found in PCOS patients were statistically significantly higher ($p < 0.001$). The areas under the curve of receiver operator characteristic plots were 0.84, 0.91, and 0.91 for T, fT, and FAI, respectively, by LC-MS/MS and 0.83, 0.90, and 0.89, respectively, by immunoassay. The intra- and inter-individual variations were 20% and 30% for T, 22% and 32% for fT, and 26% and 44% for FAI, respectively.

CONCLUSION. Apart from providing reliable reference ranges and biological variation of T, fT, and FAI in women, our data confirm the importance of taking SHBG into account when assessing androgen-status in patients evaluated for PCOS.

INTRODUCTION

Much is still unknown about the underlying mechanism of polycystic ovary syndrome (PCOS) and research is hampered by a number of factors. First, the definition of PCOS was subject to changes over the years. Currently, three diagnostic criteria prevail: the NIH criteria [1], the Rotterdam criteria [2], and the Androgen Excess and PCOS society criteria [3]. In addition, the symptoms in a population of PCOS patients are heterogeneous. Nevertheless, all criteria agree on hyperandrogenism as a key-feature in PCOS despite the absence of a consensus on the definition of hyperandrogenism. Visual scales for hirsutism, the most commonly clinical hyperandrogenic feature, are notoriously subjective [4]. Moreover, the assays used to determine serum testosterone, the most commonly measured androgen to determine biochemical hyperandrogenism, are known for their poor performance at low concentrations typically found in women

[5]. Consequently, no reliable cut-off values are available to distinguish hyperandrogenic women from women with normal androgen levels.

The introduction of more accurate methods for testosterone analysis, such as liquid chromatography-tandem mass spectrometry (LC-MS/MS) or next-generation immunoassays, may assist in reaching a consensus on the definition of hyperandrogenemia. We previously investigated the biological variation of serum total testosterone across the menstrual cycle and established reliable reference ranges for testosterone in women [6]. In the current study, using sensitive methods, we aim to determine reliable reference intervals for free testosterone (fT) and free androgen index (FAI) and test the discriminative value of these parameters in a PCOS population. In addition, the biologic variation of testosterone and testosterone-derived parameters in women was investigated.

EXPERIMENTAL SECTION

Subjects. The study protocol was reviewed and approved by an Internal Review Board for Human Studies at Massachusetts General Hospital (MGH; Boston, MA). All subjects were recruited at MGH and provided written informed consent. Reference intervals were investigated based on daily morning serum samples across a whole menstrual cycle obtained from 25 healthy women characterized by thorough physical and biochemical examination as described previously [6].

In addition, serum was obtained from 44 individuals with PCOS defined using the NIH consensus criteria [1] as oligo-ovulation (< 6 menstrual periods/yr) or anovulation in association with clinical evidence of androgen excess (i.e. hirsutism, acne, or scalp alopecia) or biochemical evidence of androgen excess (elevated levels of testosterone, initially measured at MGH), and in the absence of specific disorders of the pituitary and adrenal gland. To be enrolled in the study, subjects were required to be off hormonal therapy (birth control pills, spironolactone) for at least 3 months prior to the study and 18 – 35 years of age. In addition, subjects were not allowed to have participated in a drug study within the last 3 months. A blood sample was drawn from all PCOS study participants between 7 a.m. and 12 p.m. (noon) for routine hematology and biochemistry including hematocrit, blood urea nitrogen, creatinine, serum glutamic oxaloacetic transaminase, serum glutamic pyruvic transaminase, prolactin and thyroid-stimulating hormone. All levels were required to be within the reference range. Furthermore, a serum beta-hCG level was measured to confirm that the woman was not pregnant. An ovarian ultrasound (a transvaginal ultrasound was recommended for all women who had been sexually active and a transabdominal ultrasound was recommended for

women who had not been sexually active) was performed to quantitate the number of follicles present at the time of the blood draw.

Total testosterone measurement by ID-LC-MS/MS. The ID-LC-MS/MS protocol used to assess total testosterone was previously described in detail [6]. In short, a stable isotopically labeled internal standard (D_5-testosterone) was added to every specimen prior to work up. Testosterone was extracted with hexane/ether (4/1, v/v) and derivatized with methoxylamine hydrochloride in aqueous solution, followed by another hexane/ether extraction. HPLC separation coupled to a tandem mass spectrometric detection of a quantifier and qualifier trace for both testosterone and D_5-testosterone. 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% ($n > 20$), respectively. Accuracy was assessed by comparison between ID-LC-MS/MS and a certified reference method isotope dilution-gas chromatography-mass spectrometry (ID-GC-MS) [7]. Weighted Deming regression analysis resulted in a slope of 1.007 (0.990–1.023) (mean (95% CI)), intercept of -0.056 nmol/L (-0.100 – -0.012), and Pearson r of 0.9998 (0.9996 – 0.9999).

Total testosterone by Architect 2nd generation Testosterone. Total testosterone was also assessed by a recently available automated chemiluminescent microparticle immunoassay, the ARCHITECT® 2nd Generation Testosterone (Architect II) (Architect i2000 analyzer; Abbott Diagnostics), that was described in detail previously [6]. The limit of quantification of the assay (including undiluted measurement option) was 0.1 nmol/L (2.88 ng/dL); the assessed intra-assay variation at 0.2, 2, and 20 nmol/L was 8%, 4%, and 4%, respectively ($n > 20$), and inter-assay variation at the same concentrations was 10%, 6%, and 6%, respectively ($n > 20$).

Calculations of free Testosterone and free androgen index. For the calculations, sex hormone-binding globulin (SHBG) concentrations were measured by an Architect (Abbott Diagnostics) with an intra-assay variation in the range of 34 – 300 nmol/L of <5% and inter-assay variation at 35 and 135 nmol/L of 4.9% and 5.6%, respectively ($n > 20$). Reference range was 18 – 114 nmol/L. Free testosterone concentrations were calculated as described by Vermeulen *et al.* [8], using a standard albumin concentration of 43 g/L. The free androgen index (FAI) was calculated by the following formula: FAI = 100 × [T] / [SHBG].

Statistical Analysis. Weighted Deming regression analysis and Pearson correlation were used to compare the Architect II to ID-LC-MS/MS (Analyse-It software, Ltd., Leeds, United Kingdom). Reference intervals for free testosterone and FAI by ID-LC-MS/MS and Architect II (variables) were calculated using SPSS software (version 20). In order to minimize skew, a constant (computed by the lnskew(0) procedure in Stata 11.2 software (StataCorp, Texas, USA)) was added to each original variable before log transformation. Since the data of healthy volunteers contained repeated measurements within women, a compound symmetric covariance structure was chosen

to account for the dependency of the data. The 95% prediction interval was calculated and transformed back to obtain the reference intervals of each variable. Comparison between values found in healthy and PCOS women was done using Mann-Whitney U test (Medcalc 9.3 software, Mariakerke, Belgium). P values < 0.05 were considered to reflect statistical significance. Values are reported with their 95% confidence intervals, unless stated otherwise.

Biological variations. The biological variations for testosterone, free testosterone, and FAI in healthy women were estimated from linear mixed effects models with measurements within individuals as replicates. The total intra-individual (CV_{ti}) and total inter-individual (CV_{tg}) variations were derived using SPSS software. The intra- (CV_i) and inter-individual (CV_g) variation in healthy subjects was calculated as follows: $CV_i = \sqrt{(CV_{ti}^2 - CV_a^2)}$ and $CV_g = \sqrt{(CV_{tg}^2 - CV_a^2)}$ [9;10]. The analytical CV (CV_a) was determined by duplicate measurements of a serum sample containing 0.2 nmol/L in runs on consecutive days. The desirable performance criteria were calculated as follows: desirable $CV_a = 0.5 \times CV_i$, desirable Bias = $0.25 \times \sqrt{(CV_i^2 + CV_g^2)}$, and desirable Total Error = Bias + ($1.96 \times CV_a$) [11].

RESULTS

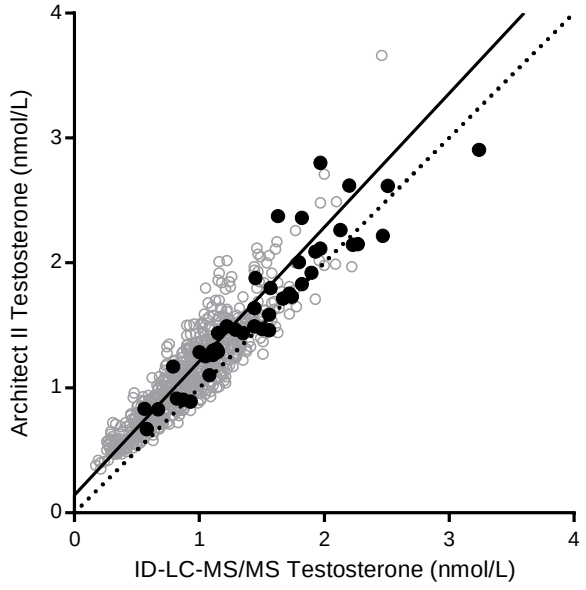

FIGURE 1: Weighted Deming regression analysis of total testosterone concentrations measured by Architect II and ID-LC-MS/MS. Open grey dots represent concentrations from healthy women as previously published [6], solid dots represent PCOS women. (n = 720, y = 1.05x + 0.14 nmol/L, r = 0.92) To convert testosterone concentrations to ng/mL, multiply by 0.288.

Testosterone was measured by two methods, ID-LC-MS/MS and Architect II. Free testosterone and the free androgen index were calculated and analyzed twice using testosterone values from both methods. There was one statistical outlier. This sample, with grossly discordant testosterone concentrations as assessed by the two methods, was

excluded from analysis and featured a testosterone concentration of 0.81 and 3.2 nmol/L by ID-LC-MS/MS and Architect II.

The 2nd generation immunoassay showed comparable accuracy in samples from healthy women as well as PCOS women (figure 1). Weighted Deming regression analysis of testosterone resulted in a slope of 1.05 (1.02 – 1.08) and intercept of 0.14 (0.12 – 0.16) nmol/L. Pearson's correlation coefficient was $r = 0.92$ (0.90 – 0.93). In general, the immunoassay overestimates testosterone values compared to the ID-LC-MS/MS. Weighted Deming regression analysis for free testosterone resulted in a slope of 1.19 (1.16 – 1.23), intercept of 0.36 (-0.07 – 0.79) pmol/L, and $r = 0.94$ (0.93 – 0.94). For FAI this was a slope of 1.25 (1.22 – 1.29), intercept of -0.04 (-0.07 – -0.01), and $r = 0.97$ (0.96 – 0.97). In all equations ID-LC-MS/MS was considered the reference method.

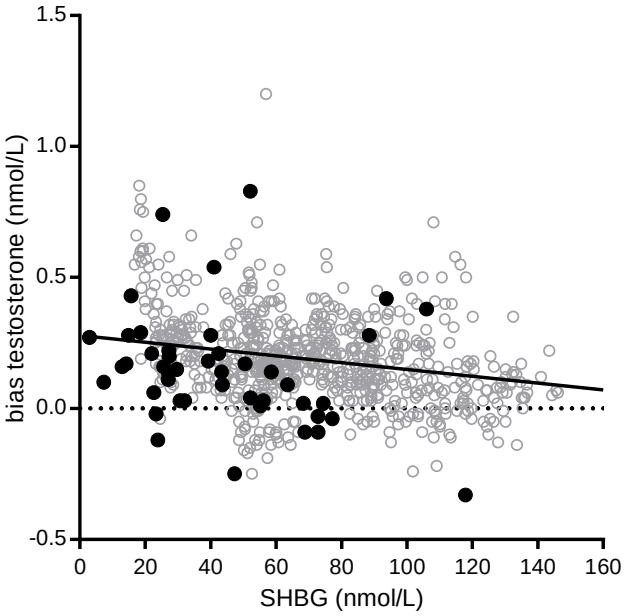

FIGURE 2: Correlation between the SHBG concentration and the absolute bias between two testosterone methods, i.e. ID-LC-MS/MS and Architect II ($n = 720$; $r = -0.267$; $p < 0.0001$). Bias was calculated by subtracting the testosterone concentration by Architect II from the testosterone concentration by ID-LC-MS/MS. Open grey dots represent values from healthy women; solid dots represent values from PCOS women.

Figure 2 demonstrates a statistically significant negative correlation between the SHBG concentration and the absolute bias between two testosterone methods ($n = 720$; $r = -0.267$ (-0.333 – -0.198); $p < 0.0001$). Furthermore, the SHBG concentration in healthy women was statistically significant higher ($p < 0.0001$) than in PCOS women, 70.7 (18.7 – 146.2) nmol/L and 41.0 (7.3 – 117.9) nmol/L (median (range)), respectively.

Table 1 summarizes the reference intervals for each parameter as well as the statistically significantly higher concentrations found in PCOS-patients. PCOS subjects were considered hyperandrogenic when a testosterone-parameter exceeded the upper reference value of the respective method.

TABLE 1: Testosterone (T), free testosterone (fT), and free androgen index (FAI) reference ranges based on healthy volunteers by ID-LC-MS/MS and Architect II and levels found in PCOS women. 1)Comparison between healthy and PCOS subjects by Mann Whitney U test. 2)The percentage of PCOS women with an elevated parameter compared to the respective reference range.

		Reference interval	PCOS median (range)	Mann-Whitney U test[1]	Hyper-androgenic[2]
T (nmol/L)	LC-MS/MS	0.3–1.6	1.5 (0.6–3.2)	$p < 0.0001$	37%
	immunoassay	0.5–2.0	1.6 (0.7–2.9)	$p < 0.0001$	30%
fT (pmol/L)	LC-MS/MS	5.2–26	31 (9–60)	$p < 0.0001$	67%
	immunoassay	7.2–33	32 (12–62)	$p < 0.0001$	44%
FAI	LC-MS/MS	0.4–2.9	3.8 (0.8–15)	$p < 0.0001$	63%
	immunoassay	0.6–4.4	4.3 (1.1–16)	$p < 0.0001$	49%

The intra- and inter-individual variation in the healthy women was estimated for testosterone, free testosterone, and free androgen index. The results are shown in table 2. Based on these biological variations, the *desirable* analytical performance for testosterone measurement should be 10.1% analytical variation (CV_a), 9.0% bias, and 28.8% total error. CV_a, bias, and total error were estimated to be 11.0 %, 9.7%, and 31.3%, respectively, for free testosterone, and 12.8%, 12.8%, and 37.8%, respectively, for FAI.

	CV_i	CV_g
T	20%	30%
fT	22%	32%
FAI	26%	44%

TABLE 2: Estimated intra-individual (CVi) and inter-individual variation (CVg) of testosterone (T), free testosterone (fT), and free androgen index (FAI) in 25 healthy women.

DISCUSSION

Daily serum samples across a menstrual cycle were collected from 25 thoroughly screened women. In this unique set of specimen total testosterone was assessed by ID-LC-MS/MS and free testosterone and free androgen index were calculated to determine reliable reference ranges and evaluate biologic variation. Furthermore, the discriminative power of these parameters was tested in serum from PCOS women. In

addition, these objectives were investigated using a 2nd generation testosterone immunoassay as well.

Only few data on biological variation of testosterone parameters in women have been reported. This might be because the most commonly used assays for measuring testosterone lack sensitivity and accuracy at concentrations typically found in women. Recently, more sensitive methods such as ID-LC-MS/MS and 2nd generation immunoassays have been introduced in the clinical laboratory. In order to apply these methods on samples from women in clinical practice, the performance characteristics should meet the criteria based on the biological variation [9]. The estimated biological variation for women derived from our data is in accordance with the variations published by Garde and colleagues [12]. The variations are, however, generally greater than the biological variations reported for men [13-16]. Consequently, the *desirable* analytical performance criteria for testosterone measurement may be less stringent when measuring in samples from women. The biological variations estimated from this study illustrates a high degree of individuality, as reported in men [9], and limits the use of a single measurement.

Hyperandrogenism is one of the key-features of PCOS and as expected, in our study, the majority of PCOS women show significantly elevated testosterone concentrations, calculated free testosterone concentrations, and free androgen index. The percentage of PCOS-patients with testosterone and free testosterone concentrations by ID-LC-MS/MS above the upper limit of the reference values is similar to that reported by Chang *et al.* [17]. The percentage of PCOS women that were considered biochemically hyperandrogenic based on one of the testosterone parameters revealed that the discriminative value of free testosterone and FAI was better than serum total testosterone. This is in agreement with other reports [18-20]. It is important to realize that the variation of not only testosterone assays but also the variation of the SHBG assay should be taken into account in these parameters. There seems to be great difference in the calibration of SHBG assays [21]. In addition, multiple algorithms for the calculation of free testosterone have been described [22]. Therefore, the reference intervals should be adapted taking into account the method of choice, *i.e.* algorithm, testosterone concentration, and SHBG concentration. Azziz *et al.* [3] showed in an overview of six published studies that there are large differences in prevalence of hyperandrogenemia among PCOS patients (22 to 81% for elevated total testosterone). This might be because of the different assays that are used for measuring testosterone as illustrated by our data, *i.e.* the different percentage of hyperandrogenic PCOS women when testosterone is measured by ID-LC-MS/MS or immunoassay.

Different studies have recommended measurement of testosterone during the early follicular phase for the diagnosis of PCOS [24;25]. We previously reported that testosterone concentrations are statistically significantly elevated around ovulation, however the day-to-day variation in individuals is higher and exceeds the elevated group

average [6]. By analyzing the free testosterone and FAI for each individual across the menstrual cycle, we observed the same fluctuations as previously described for total testosterone. The intra-individual variation appears to be independent on the day of the menstrual cycle for the measurement of testosterone, free testosterone, and FAI in PCOS. Therefore, in our opinion, the moment of blood sampling is not important.

In addition to ID-LC-MS/MS data, testosterone concentrations in the same samples were also determined by the Architect 2nd generation immunoassay. A consistent positive bias was reported previously in healthy women [6] and in this study the bias was also observed in serum from PCOS women. This resulted in absolute higher reference intervals for all testosterone parameters measured by the immunoassay than by the ID-LC-MS/MS. Moreover, a better correlation between ID-LC-MS/MS and Architect II derived testosterone parameters was found when SHBG was taken into account. The 2nd generation immunoassay was influenced by the presence of SHBG, analogous to vitamin D immunoassays that are influenced by vitamin D binding protein [26]. The biological significance of this observation, if any, remains to be established. The immunoassay tends to show less bias or even a negative bias for samples with high SHBG concentrations. This indicates that testosterone is not sufficiently released from SHBG when the concentration of this testosterone-binding protein is increased. These samples might benefit from extra manual sample preparation, such as liquid extraction [5] Also, the bias was relatively low in samples from women with PCOS, this might be because of the combination of relatively low SHBG and high testosterone concentrations. The contribution of the SHBG effect to the already established bias between the ID-LC-MS/MS and the immunoassay remains to be resolved.

Despite the high degree of comparability between the ID-LC-MS/MS and the second generation immunoassay, the ID-LC-MS/MS allows identification of a higher proportion of hyperandrogenemia in PCOS patients. Since the diagnosis is generally not based on measurement of testosterone and SHBG alone, the clinical impact of this difference requires further investigation. Nevertheless, taking the performance criteria based on biological variability into account, our data indicates that the 2nd generation immunoassay is meets the criteria of desirable analytical variation to measure testosterone concentrations in women in clinical practice. The previously reported bias, i.e. 21.3% (-12.7 to 55.3% (mean (95% confidence interval)) [6], is greater than the desired goal. Therefore, testosterone concentrations measured by Architect II should be interpreted relative to the method-specific reference intervals.

CONCLUSION

Apart from providing reliable reference ranges and biologic variation in women, our data confirm the importance of taking SHBG into account when assessing androgen status in patients evaluated for PCOS. In this study, no clear difference was found between the diagnostic value of calculated free testosterone and FAI in PCOS women. According to our data, testosterone measurement by ID-LC-MS/MS and Architect 2nd generation immunoassay show comparable performance when it comes to distinguishing between PCOS and normal women.

REFERENCES

[1] Zawadzki JK, Dunaif A. Diagnostic criteria for polycystic ovary syndrome: towards a rational approach. In: Dunaif A, Givens JR, Haseltine FP, Merriam GR, eds. Polycystic ovary syndrome. Oxford, England: Blackwell Scientific, 1992:377-84.

[2] Rotterdam ESHRE/ASRM-sponsored PCOS consensus workshop group. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS). Hum Reprod 2004;19:41-7.

[3] Azziz R, Carmina E, Dewailly D, Diamanti-Kandarakis E, Escobar-Morreale HF, Futterweit W et al. Positions statement: criteria for defining polycystic ovary syndrome as a predominantly hyperandrogenic syndrome: an Androgen Excess Society guideline. J Clin Endocrinol Metab 2006;91:4237-45.

[4] Wild RA, Vesely S, Beebe L, Whitsett T, Owen W. Ferriman Gallwey self-scoring I: performance assessment in women with polycystic ovary syndrome. J Clin Endocrinol Metab 2005;90:4112-4.

[5] Tiel Groenestege WM, Bui HN, Ten Kate J, Menheere PP, Oosterhuis WP, Vader HL et al. Accuracy of First and Second Generation Testosterone Assays and Improvement through Sample Extraction. Clin Chem 2012;58:1154-6.

[6] Bui HN, Sluss PM, Blincko S, Knol DL, Blankenstein MA, Heijboer AC. Dynamics of serum testosterone during the menstrual cycle evaluated by daily measurements with an ID-LC-MS/MS method and a 2nd generation automated immunoassay. Steroids 2013;78:96-101.

[7] Thienpont LM, van Uytfanghe K, Blincko S, Ramsay CS, Xie H, Doss RC et al. State-of-the-art of serum testosterone measurement by isotope dilution-liquid chromatography-tandem mass spectrometry. Clin Chem 2008;54:1290-7.

[8] Vermeulen A, Verdonck L, Kaufman JM. A critical evaluation of simple methods for the estimation of free testosterone in serum. J Clin Endocrinol Metab 1999;84:3666-72.

[9] Yun YM, Botelho JC, Chandler DW, Katayev A, Roberts WL, Stanczyk FZ et al. Performance Criteria for Testosterone Measurements Based on Biological Variation in Adult Males: Recommendations from the Partnership for the Accurate Testing of Hormones. Clin Chem 2012;58:1703-10.

[10] Fraser CG, Harris EK. Generation and application of data on biological variation in clinical chemistry. Crit Rev Clin Lab Sci 1989;27:409-37.

[11] Fraser CG, Hyltoft PP, Libeer JC, Ricos C. Proposals for setting generally applicable quality goals solely based on biology. Ann Clin Biochem 1997;34:8-12.

[12] Garde AH, Hansen AM, Skovgaard LT, Christensen JM. Seasonal and biological variation of blood concentrations of total cholesterol, dehydroepiandrosterone sulfate, hemoglobin A(1c), IgA, prolactin, and free testosterone in healthy women. Clin Chem 2000;46:551-9.

[13] Ricos C, Arbos MA. Quality goals for hormone testing. Ann Clin Biochem 1990;27:353-8.

[14] Maes M, Mommen K, Hendrickx D, Peeters D, D'Hondt P, Ranjan R et al. Components of biological variation, including seasonality, in blood concentrations of TSH, TT3, FT4, PRL, cortisol and testosterone in healthy volunteers. Clin Endocrinol (Oxf) 1997;46:587-98.

[15] Ahokoski O, Virtanen A, Huupponen R, Scheinin H, Salminen E, Kairisto V, Irjala K. Biological day-to-day variation and daytime changes of testosterone, follitropin, lutropin and oestradiol-17beta in healthy men. Clin Chem Lab Med 1998;36:485-91.

[16] Andersson AM, Carlsen E, Petersen JH, Skakkebaek NE. Variation in levels of serum inhibin B, testosterone, estradiol, luteinizing hormone, follicle-stimulating hormone, and sex hormone-binding globulin in monthly samples from healthy men during a 17-month period: possible effects of seasons. J Clin Endocrinol Metab 2003;88:932-7.

[17] Chang WY, Knochenhauer ES, Bartolucci AA, Azziz R. Phenotypic spectrum of polycystic ovary syndrome: clinical and biochemical characterization of the three major clinical subgroups. Fertil Steril 2005;83:1717-23.

[18] Barth JH, Field HP, Yasmin E, Balen AH. Defining hyperandrogenism in polycystic ovary syndrome: measurement of testosterone and androstenedione by liquid chromatography-tandem mass spectrometry and analysis by receiver operator characteristic plots. Eur J Endocrinol 2010;162:611-5.

[19] Escobar-Morreale HF, Asuncion M, Calvo RM, Sancho J, San Millan JL. Receiver operating characteristic analysis of the performance of basal serum hormone profiles for the diagnosis of polycystic ovary syndrome in epidemiological studies. Eur J Endocrinol 2001;145:619-24.

[20] Cho LW, Kilpatrick ES, Jayagopal V, Diver MJ, Atkin SL. Biological variation of total testosterone, free androgen index and bioavailable testosterone in polycystic ovarian syndrome: implications for identifying hyperandrogenaemia. Clin Endocrinol (Oxf) 2008;68:390-4.

[21] de Ronde W, van der Schouw YT, Pols HA, Gooren LJ, Muller M, Grobbee DE, de Jong FH. Calculation of bioavailable and free testosterone in men: a comparison of 5 published algorithms. Clin Chem 2006;52:1777-84.

[22] Sartorius G, Ly LP, Sikaris K, McLachlan R, Handelsman DJ. Predictive accuracy and sources of variability in calculated free testosterone estimates. Ann Clin Biochem 2009;46:137-43.

[23] Knochenhauer ES, Key TJ, Kahsar-Miller M, Waggoner W, Boots LR, Azziz R. Prevalence of the polycystic ovary syndrome in unselected black and white women of the southeastern United States: a prospective study. J Clin Endocrinol Metab 1998;83:3078-82.

[24] Salonia A, Pontillo M, Nappi RE, Zanni G, Fabbri F, Scavini M et al. Menstrual cycle-related changes in circulating androgens in healthy women with self-reported normal sexual function. J Sex Med 2008;5:854-63.

[25] Barth JH, Yasmin E, Balen AH. The diagnosis of polycystic ovary syndrome: the criteria are insufficiently robust for clinical research. Clin Endocrinol (Oxf) 2007;67:811-5.

[26] Heijboer AC, Blankenstein MA, Kema IP, Buijs MM. Accuracy of 6 routine 25-hydroxyvitamin D assays: influence of vitamin D binding protein concentration. Clin Chem 2012;58:543-8.