MANAGEMENT OF ENDOCRINE DISEASE

Hyperandrogenic states in women: pitfalls in laboratory diagnosis

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Abstract

Measuring total testosterone level is the first-line approach in assessing androgen excess in women. The main pitfalls in measuring testosterone relate to its low concentration and to the structural similarity between circulating androgens and testosterone, requiring accurate techniques with high specificity and sensitivity. These goals can be achieved by immunoassay using a specific anti-testosterone monoclonal antibody, ideally after an extraction step. Liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) will be commonly used for measuring testosterone, providing optimal accuracy with a low limit of detection. Yet, the pitfalls of these two techniques are well identified and must be recognized and systematically addressed. In general, laboratories using direct testosterone immunoassay and mass spectrometry need to operate within a quality framework and be actively engaged in external quality control processes and standardization, so as to ensure appropriate interpretation irrespective of the particular laboratory. Circulating testosterone is strongly bound to sex-hormone-binding globulin (SHBG), and SHBG levels are typically low in overweight hyperandrogenic patients. Thus, low SHBG may decrease circulating testosterone to normal values, which will mask androgen excess status. One way to avoid this pitfall, awaiting direct free testosterone assays that are yet to be developed, is to measure SHBG and calculate free testosterone. A few other pitfalls will be discussed in this review, including those of adrenal androgen exploration, with the aim of helping clinicians to better handle laboratory investigation of androgen excess disorders in women.

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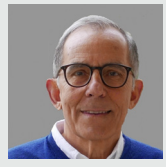
Invited Author's profile

Michel Pugeat is currently Emerit Professor, University Claude Bernard, Lyon, France. The main areas of Prof. Pugeat's research is on sex steroid hormones and their binding proteins with the original description of phenotypes associated to *CBG/SHBG* gene mutations and the evidence that SHBG/CBG levels are associated with metabolic syndrome and its inherent risks for developing chronic diseases. His research specifically focuses on the laboratory investigation of polycystic ovary syndrome (PCOS) emphasizing that SHBG does influence the clinical interpretation of total testosterone. He has undertaken prospective research on the endocrine disrupter bisphenol A (BPA) and in developing a highly sensitive BPA immunoassay that has been a useful tool for investigation of disease related to environment. His current interest is on the influence of androgens on sexual arousal and their deviance in males.



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Introduction

Hyperandrogenic states are the clinical manifestation of excess androgen disorder in women. They encompass excess androgen production associated with exceptional androgen-secreting tumors, rare genetic diseases that impair adrenal steroidogenesis, and the very common polycystic ovary syndrome (PCOS) (1, 2, 3, 4, 5, 6). Hyperandrogenic states also include increased peripheral androgen metabolism and clearance that enhance androgen cell availability and bioactivity (7, 8). Finally, they may also involve increased androgen activity within the cell, through active intracellular metabolism and/or enhanced androgen receptor machinery (9). Overall prevalence of hyperandrogenic states is significant, at 6–12% worldwide (10, 11) and raises the question of how laboratory investigations should be conducted for diagnosis (12, 13, 14).

Endocrine diseases are typically suspected on clinical symptoms, a combination of which suggests excess or deficient hormonal secretion and/or action. Excessive or deficient endocrine gland activity is associated with activation or inhibition of the specific regulatory system maintaining endocrine function homeostasis and/or limiting the consequences of surplus or deficiency in the considered hormone (15). This robust paradigm of feedback regulation assumes that it is usually possible to identify most endocrine disorders by measuring hormones from thyroid, adrenal and gonadal sources and their specific pituitary regulatory factors, in blood, saliva or urine samples or even within cells. However, although this paradigm is well adapted to laboratory thyroid investigation, balancing thyroid hormone (T4/T3) and thyroid-stimulating hormone (TSH) data, it is less consistent when applied to female androgen disorder: changing hormonal profile during the menstrual cycle, the positive rather than negative feedback of estradiol regulation on luteinizing hormone (LH) regulation and the inhibitory effect of androgens on LH inhibitory regulation of progesterone have been puzzling for consensual recommendations on how to evaluate gonadotropin profile in androgen excess women (16, 17).

This review will challenge the concept that androgen investigation can accurately identify the origin of hyperandrogenic states in females, by identifying the pitfalls and how to circumvent them so as finally to succeed in diagnosis.

Consensus and recommendations for the diagnostic approach to hyperandrogenic states

Hyperandrogenic state is identified on excessive hair growth with a male pattern (hirsutism) and is suspected in patients with recurrent acne with seborrhea or androgenetic alopecia (9). Rapid onset of virilizing symptoms such as voice masculinization, abnormal muscle development and clitoris enlargement are suggestive of rare androgen-secreting tumors and must be promptly identified (5, 9).

Most consensus statements recommend measuring total testosterone as first-line investigation of hyperandrogenic states (Figure 1). These include the 1990 NIH-sponsored conference on PCOS (2, 14), the Rotterdam ESHRE/ASRM-sponsored PCOS consensus workshop group (3), the Androgen Excess and PCOS Society (4), the French Endocrine Society Consensus (13) and the PCOS Special Interest Group of the European Society of Endocrinology (18).

Is total testosterone the relevant hormone for identifying hyperandrogenic states? Testosterone circulates in the blood, loosely bound to albumin but tightly and specifically bound to sex hormone-binding globulin (SHBG). According to the law of mass action, a small steroid fraction is protein-unbound and available for target cells; in women, this fraction is less than 2%, while the albumin-bound complex is 41% and the SHBG-bound complex is 57% (19). There is no definitive

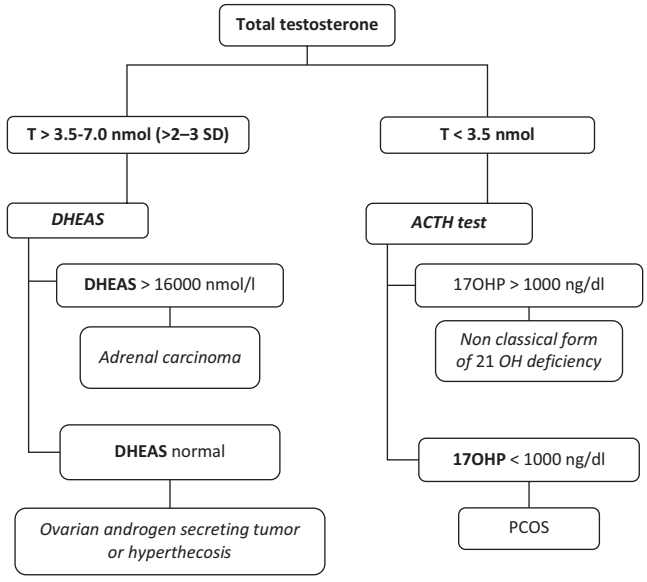

Figure 1
Paradigm for identifying androgen excess in women.

evidence for a specific transit of the SHBG-bound steroid complex, although it has been shown that SHBG, by interacting with megalin, the main endocytic receptor (20), may provide a specific pathway for cellular uptake of biologically active SHBG-bound androgens and estrogens. This has been reported for trophoblastic cell lines and might be an important pathway for androgen transport to the placenta for aromatization during pregnancy. Moreover, human SHBG is found in human breast and prostate cancer cells (21) and, by interacting with fibulin, a matrix-associated protein, can be sequestered from the blood into the stromal matrix of the uterine endometrium (22). Taken together, these intriguing findings challenge the free-hormone hypothesis (see below).

The rationale for measuring testosterone is essentially based on the assumption that testosterone is the main active circulating androgen. Testosterone has a strong 'media profile' for the public, students and physicians. It is primarily produced as an androgen precursor: androstenedione from thecal cells and dehydroepiandrosterone (DHEA) from the adrenal cortex (23), which are converted into testosterone, especially in adipose tissue. The production rate (PR) of testosterone, including direct ovarian secretion and peripheral metabolism from both ovarian and adrenal precursors, maintains circulating testosterone concentration. Conversely, the metabolic clearance rate (MCR), defined as the volume of blood cleared irreversibly per unit of time, is the main process decreasing circulating androgen concentrations. The strong binding affinity of SHBG to dihydrotestosterone, testosterone and estradiol (in decreasing order of magnitude (19)) is inversely correlated to their respective MCRs, which are 315, 485 and 720 L/day respectively (23, 24, 25, 26). In contrast, DHEA and androstenedione show low binding affinity and bind weakly to SHBG, with much higher MCRs of 2200 and 1200 L/day respectively (Table 1).

Table 1 Half-life ($T^{1/2}$), metabolic clearance rate (MCR) and binding affinity constant at 37°C according to Longcope *et al*. (23, 24), Mahoudeau *et al*. (25) and Dunn *et al*. (19).

	$T^{1/2}$ (min)	MCR (L/day)	SHBG binding affinity*
Dihydrotestosterone	53	315	5.5
Testosterone	34	485	1.2–1.3
Estradiol	ND	720	0.7
Androstenedione	50	2200	0.03
Dehydroepiandrosterone	60	2040	0.07

*K_a: $10^{-9} \times M^{-1}$ at 37°C.

Pitfalls in measuring total testosterone by direct immunoassay

Testosterone level is poorly correlated with the severity of hirsutism. This point has been documented using a reference testosterone assay (27). Severely hirsute patients may have normal testosterone levels, suggesting high androgen sensitivity or increased testosterone clearance. Conversely, the Rotterdam consensus (3) pointed out that patients with polycystic ovarian morphology on ultrasound may show increased testosterone levels without increased hair growth or recurrent acne.

Variations in testosterone levels over the menstrual cycle are fairly insignificant. Although it is recommended that testosterone be measured in the early follicular phase, in practice, blood sampling in fasting conditions at any time of day or point in the cycle is effective for measuring testosterone.

Low testosterone concentration and the structural similarity between circulating androgens and testosterone require accurate and sensitive techniques (28). This can be achieved by competitive immunoassays that rely on the inherent ability of an antibody to bind small molecules. To assay testosterone, antibodies are developed that match its particular structure, at the price of their specificity, as the configuration of testosterone is similar to that of other circulating androgens. An extraction step, using an organic solvent, followed by purification of the extract by chromatography (Celite, HPLC, Sephadex LH20), eliminates steroids likely to interfere with the immunological reaction because of their similar structure and/or insufficient antibody specificity (12, 13). Steroid extraction also eliminates much of the matrix effect, mainly related to steroid transport proteins (albumin and SHBG).

Precision and specificity are mainly dependent on the quality of the antibodies used to capture testosterone and on the technique used to reveal the antibody-bound testosterone complex.

The low limit of detection or sensitivity of the immunoassay is in part dependent on the labeled probe and the system used to immobilize the immune complex. The chief markers are radioactive (iodine 125 and tritium), enzymatic (HRP and PAL), fluorescent (europium) and chemiluminescent. Steroid immunoassays using time-resolved fluorometric detection of europium (Delfia Technology) usually exhibit higher sensitivity than tritiated or enzyme-conjugated tracers, and challenge immunoassays using 125-iodine-labeled steroids, with the advantages of a stable non-radioactive tracer (29).

Table 2 Main advantages, disadvantages and pitfalls with direct immunoassay.

Advantages	Disadvantages and pitfalls
• RIAs and chemiluminescence immunoassays are the most widely used methods (>80% of labs) • Easy to use, requiring small sample volume, short assay time • Suitable for automated platforms that reduce the risk of human error • Precision: good intra- and inter-assay reproducibility • Increased effort by manufacturers to calibrate their assays	• Low accuracy • Lack of specificity, which depends on the quality of testosterone antibodies • Potential interference with binding proteins • Potential interference and matrix effects, despite good specificity of the antibodies used • Unexpected interference: e.g., biotin

In addition, immunoassays exploit the high-affinity streptavidin-biotin system by incorporating the small biotin molecule in enzymes or in the hormone to be measured. The high binding affinity ($K_a = 10^{15}$) of streptavidin for biotin and biotinylated compounds is exploited to immobilize immune complexes in the solid-phase matrix. This approach has considerably enhanced the precision of immunometric assays.

Nowadays, chemiluminescence and fluorescence are the major detection principles in modern automated analyzers, whereas radioactive systems are less widely used. The main advantages, disadvantages and pitfalls of measuring testosterone by direct immunoassay are listed in Table 2.

Novel pitfalls associated with advances in hormone immunoassay

Hormone immunoassays using the streptavidin-biotin system can be affected by high levels of circulating biotin, leading to a risk of misdiagnosis. This risk of false diagnosis has long been recognized for thyroid diseases (30, 31, 32, 33). Biotin (vitamin B7) is a water-soluble vitamin with a recommended daily intake (RDI) in the order of milligrams per day. High-dose biotin (10 000 times the RDI) is currently indicated to improve clinical outcome and quality of life in patients with progressive multiple sclerosis. An excess of biotin in the blood sample, by competition with the trace of biotinylated hormone or the biotinylated specific antibody, depending on the system used, will bind to streptavidin-coated microparticles in the solid phase and interfere with the signaling system, leading to over- or under-estimation. To overcome this pitfall, it has been shown that adsorption of biotin in magnetic streptavidin-coated microparticles efficiently eliminates biotin interference, when included in assays kits (34). A potential benefit has been claimed for biotin treatment in various diseases, including alopecia and diabetes mellitus, and patients may sometimes use biotin as a complementary medicine. In these conditions, false high results for testosterone and DHEAS have been reported (33), but this remains to be further documented.

Measuring testosterone by mass spectrometry: a must

This chapter refers to comprehensive reviews (35, 36, 37, 38, 39, 40) that readers are encouraged to consult for more information on mass spectrometry principles, components and software.

Gas chromatography (GC) coupled with MS was the initial analytical approach for simple molecular-mass-selective detection and is the reference method for measuring testosterone (35, 36). GC–MS requires a large blood sample (>2 mL) for a step of extraction and derivatization. The development of liquid chromatography (LC) and the emergence of atmospheric pressure chemical ionization and of electrospray ionization has allowed direct coupling of LC and MS. LC–MS constitutes a remarkable progress, since the eluent phase is liquid, making derivatization unnecessary for measuring most steroids, including testosterone, which possess unsaturated carbonyls that are readily ionized without derivatization.

The introduction of tandem MS, which involves coupling 2 quadrupole mass filters with an interposed collision cell to reveal the fragmentation pattern of target steroids, has greatly increased the selectivity of LC–MS. Presently, LC–MS/MS offers the best quality for steroid measurement. It has substantially improved our current approach to and understanding of congenital steroid metabolism disorders, including 21-hydroxylase deficiency (37).

Although LC–MS/MS is not widely used in laboratories in Europe, because the high cost of the equipment, the special expertise required and the time-consuming procedure make large series of assays impractical, it may in the near future be suitable for routine use, as costs decrease, becoming an alternative to immunoassay when automated analysis platforms are available.

Ultra-performance liquid chromatography (UPLC) is now enhancing analysis speed, sensitivity and resolution (38). The precision and accuracy of LC–MS/MS and its low limits of detection argue for it being the reference for assessing androgen status in women and children (28, 36).

Pitfalls in measuring testosterone by mass spectrometry

Application of LC–MS/MS should provide accurate measurement of total testosterone levels. Yet, pitfalls must be recognized and systematically addressed (38, 39). The selectivity of MS/MS detection was originally overestimated. The inaccuracy of LC–MS/MS methods may be related to the ionization process and matrix effects with differential influence on target analytes and internal standard compounds. Most potential sources of inaccuracy can be controlled by sufficient LC separation-based sample workup before MS analysis.

Finally, important sources of unreliable results include human error, the risk of which can likely be minimized by automated clinical chemistry analyzers. Only a few commercial LC–MS/MS assay kits are presently available, and their instrument configurations are extremely still heterogeneous. Automating the LC–MS/MS processes, with the final goal of developing fully automated MS/MS-based analyzer systems, is not only a prerequisite for more widespread use of this powerful technology in clinical laboratories but also essential to increase reliability. The main advantages and drawbacks of LC–MS/MS for measuring testosterone are listed in Table 3.

Current recommendations for total testosterone assay

The current position advocates appropriate use of both immunoassay and mass spectrometry-based methods for measuring total testosterone for the diagnosis of hyperandrogenic states in women. This realistic approach was defended by Taylor *et al*. (40), echoing Franz Kafka's philosophy: 'Start with what is right rather than what is acceptable'. With this aim in view, LC–MS/MS allows highly accurate analyses with enhanced specificity. However, pitfalls must be recognized and addressed, and they equally echo Voltaire's maxim that 'The best is the enemy of good', arguing that replacing all immunoassays by MS technology would be 'an unrealistic and unnecessary goal', because the availability of MS is limited by cost and technical demands. Recommendations for measuring testosterone can realistically be founded on this attitude. An ultimate consensus will be possible when MS equipment becomes available all around the world (3, 4, 13, 18).

The free-hormone hypothesis

The free-hormone hypothesis (41, 42) states that the protein-unbound or free circulating hormone fraction is bioactive. There is good evidence that free-hormone concentration reflects the clinical situation more accurately than total plasma hormone level. This concept has been widely developed for routine investigation of thyroid function, to allow for changes in thyroid hormone-binding globulin (TBG) level, notably during pregnancy and under oral contraception.

Similar considerations should be applied to SHBG, the main transport system for testosterone and estradiol, which modulates their bioactivity by restrictive diffusion into target tissue. The free-hormone hypothesis has been re-validated on a mouse model overexpressing *h*SHBG (43). In this model, a remarkable increase in circulating SHBG was associated with prolonged half-life of SHBG-bound testosterone and consequently increased testosterone concentration. In contrast, free testosterone was essentially unchanged, likely the consequence of adaptive feedback regulation with a significant increase in LH level. Nevertheless, male transgenic mice exhibited a mild hypogonadal phenotype that was possibly due to low bioavailability of sex steroid hormone to target cells, as shown using tritium-labeled probes. These results strongly substantiate the concept that a key physiological function of SHBG is to maintain testosterone levels and to regulate cell bioavailability. This model is translatable to human male physiology, where feedback regulation of gonadotropin maintains free testosterone levels within the normal range.

In women, however, androgens show minimal if any negative feedback regulation of LH secretion (44). In

Table 3 Main advantages vs disadvantages and pitfalls with LC–MS/MS for measuring testosterone.

Advantages	Disadvantages and pitfalls
• Accuracy • High specificity owing to its ability to select for the mass of the compound of interest (parent ion) and to fragment the parent ion into specific, smaller ions (daughter ions) • Sensitivity • Potential for simultaneous measurement of several different steroids	• Expensive equipment • Technical demands • Variability between labs (risk of human error, automata are not yet available) • Technical pitfalls: high degree of variation in the efficiency of atmospheric pressure ionization, standardization mandatory, 'isotope effects' of internal standards, differential impact of matrix effects on analyte and isotope internal standard, interference from in-source transformation of conjugate metabolites, isobaric congeners, matrix compounds sharing mass transitions, etc.

contrast, the positive feedback regulation of LH pulsatile secretion by estradiol, observed in the late follicular phase (44), may increase LH levels and maintain androgen excess. This is especially the case in patients with low SHBG, which contributes *per se* to clinical hyperandrogenism. It is well documented that insulin resistance (45), liver lipogenesis and/or inflammation (46, 47) decrease SHBG levels by a well-identified mechanism in the liver expression of SHBG gene. In addition, it is also documented that androgen excess reduces the inhibition of gonadotropin-releasing hormone pulse frequency normally exerted by progesterone, causing rapid LH pulse secretion and further maintaining or increasing ovarian androgen production (48). Thus, increased LH secretion maintains high ovarian androgen production, in a vicious circle that was early described by Samuel Yen (49). The current concept is that androgen excess promotes abnormal neuroendocrine LH secretion, which in turn perpetuates the hyperandrogenic state (48). It is noteworthy that administration of flutamide, a non-steroid androgen receptor blocker, has essentially no effect on gonadotropin regulation in normal women (50) but restores some progesterone feedback sensitivity in adolescent PCOS subjects, with decreased LH pulsatility (51).

Measuring or calculating free testosterone concentration

According to the free-hormone hypothesis, measuring the protein-unbound fraction of testosterone provides a better index of overall production and a marker of biological activity (41, 42). The technical challenge is to develop a methodology that does not disturb the binding equilibrium between steroids and binding proteins, and accurate and highly sensitive assays for measuring the free concentration, which is much lower than total testosterone.

Equilibrium dialysis has long been the standard method to separate protein-bound from free testosterone prior to quantification (52) and is considered to provide the best estimate of free testosterone plasma concentration, despite technical difficulties (53). By adding a trace of ^{3}H-testosterone to the serum sample, with an appropriate incubation time at 37°C, the SHBG- and albumin-bound testosterone fractions can be separated from the free testosterone through a membrane in a small dialysis tube or chamber. This method allows measurement of the percentage of free ^{3}H-testosterone, which is usually 2–3% of the total testosterone circulating in the blood. It also allows measurement of the actual concentration of free testosterone in the dialysate, using a reliable testosterone assay. It requires highly purified radioactive 3H-testosterone as tracer (54, 55).

Centrifugal ultrafiltration dialysis has been validated and found to be faster, with fewer technical requirements than equilibrium dialysis (56). A reference method, coupling ultrafiltration to GC–MS detection, has been recently developed (57) with further improvement in analytical sensitivity, convenience and sample requirements when coupled with LC–MS/MS (58).

Awaiting routine availability of these promising techniques, clinicians must bear in mind that the current commercially available immunoassays for free testosterone show inadequate accuracy and precision (59).

Surrogate methods to calculate free testosterone concentration

Calculation of free testosterone from testosterone and SHBG concentrations using accurate assays has been claimed to be a reliable approach for routine clinical practice (60). Using the laws of mass action, it provides a surrogate for direct free testosterone measurement, but is dependent on the equation that is used (61, 62). The calculation assumes that there is no inter-individual variability in SHBG-binding affinity and that albumin concentrations and albumin-binding affinity for testosterone are constant. It also considers that interaction with endogenous ligands that may significantly displace testosterone from SHBG-binding sites is negligible. Various derived testosterone calculations to estimate free testosterone have been proposed, including a free testosterone index (FTI), defined as the ratio of total testosterone to SHBG, expressed as a percentage. Theoretically, FTI corresponds to free testosterone when the molar ratio of total testosterone to SHBG is low, which is the case in women but not in adult men (61, 62). In general, calculated values for free testosterone correlate well with equilibrium dialysis results (60, 63).

Pitfalls in calculating free testosterone should be recognized. SHBG gene polymorphism, associated with change in SHBG-binding affinity, has been identified (64) and one study reported an effect of SHBG concentration on apparent affinity (65). More importantly, the Dunn model of the binding equilibrium of natural endogenous ligands (19) provides evidence that potential SHBG binders, such as drugs or endocrine disrupters, may disturb SHBG-binding sites and consequently the free-hormone fraction (66).

Bioavailable testosterone encompasses albumin-bound and SHBG-bound testosterone and has been claimed to

be the bioavailable fraction of circulating testosterone for target cells (67). The use of saturated ammonium sulfate to precipitate the globulins, including SHBG, before centrifugation to separate SHBG-bound ^{3}H-testosterone from albumin-bound plus free ^{3}H-testosterone, has been reported to be effective in a few laboratories (68). A major limitation of the ammonium sulfate precipitation assay method is incomplete precipitation of globulins, including SHBG, which may increase intra- and inter-assay variability. With some technical precautions, non-SHBG-bound testosterone is a reliable instrument for routine evaluation of androgen excess (69, 70).

Free vs total testosterone in hyperandrogenic patients

Most studies reported in the literature calculated free testosterone from total testosterone, SHBG or albumin, according to Vermeulen *et al.* (53). In a consecutive population of clinically hyperandrogenic patients, Azziz *et al.* (71) reported that overall total testosterone was elevated in 38% of cases, while for free testosterone was increased in 55.5%; in this study, one-fifth of patients had normal androgen levels. In a consecutive series of hirsute patients, Carmina *et al.* (72) reported PCOS on the Rotterdam criteria in two-thirds of patients, total testosterone being the most commonly elevated androgen; free testosterone was similarly discriminating for PCOS diagnosis, but not superior. Interestingly, one study showed that serum SHBG levels showed good sensitivity and specificity (87%) for diagnosis of PCOS (73). Using a MS-based technique, Stener-Victorin *et al.* reported that free testosterone concentration was useful for PCOS diagnosis, but that estrone concentration, alone or in combination to elevated free testosterone, was more discriminating (74).

Alternative approach: measuring androstenedione

O'Reilly *et al.* (75) reported, in PCOS patients on the Rotterdam criteria, that high testosterone levels correlated with high androstenedione and free androgen index; however, high androstenedione level was a more sensitive marker in PCOS-related androgen excess. They also reported a strong negative association between serum androstenedione and insulin sensitivity, and a high incidence of glucose intolerance correlating with the severity of the androgen phenotype. Interestingly, Lerchbaum *et al.* (76) reported that elevated free testosterone, unlike isolated androstenedione elevation, was associated with an adverse metabolic phenotype.

The combination of total testosterone, androstenedione and free testosterone provides good accuracy for diagnosis of PCOS and its hyperandrogenemic sub-phenotypes (76, 77, 78). However, application for predicting metabolic risk in PCOS needs further investigation (79).

Adrenal androgen excess investigation

The adrenal cortex produces *DHEA* in the zona reticularis under the control of adrenocorticotropic hormone (ACTH), with no evidence of negative feedback regulation of pituitary ACTH secretion by DHEA. DHEA has a short half-life of ~50 min, with high clearance of 2040 L/day (80). A significant portion of DHEA is sulfated through the action of DHEA sulfotransferase (81). DHEAS is strongly bound to albumin and is cleared from the circulation at a very slow rate of 12.8 L/day, with a long half-life of ~17 h (80). Consequently, in women, DHEAS concentration is relatively stable throughout the day (82). DHEAS is the precursor of active androgens and can be taken up by ovarian follicles to synthesize testosterone (83) and even converted into dihydrotestosterone in peripheral tissues once converted to androstenedione, without requiring prior formation of testosterone (84).

DHEA production varies with aging and starts decreasing after 30 years (85), with no change in ACTH secretion. This aging profile of androgen secretion might be associated to decline activity in selected enzymatic activities, such as cytochrome-b5-dependent 17,20 lyase (CYP17), in the reticularis zone of the adrenal gland (85), but remains to be further investigated. SULT2A1 is the major enzyme responsible for DHEA sulfation in the adrenal glands and liver. The sulfate donor PAPS is required for sulfotransferase activity. In humans, PAPS is synthesized by the two isoforms of PAPS synthase, PAPSS1 and PAPSS2. Sulfation of DHEA to DHEAS is the major pathway of DHEA metabolism, suggesting that increased DHEA sulfation may limit the amount of DHEA available for androgen synthesis. By contrast, mutations of the gene encoding human PAPS synthase 2 (*PAPSS2*) have been reported in patients with very low DHEAS levels but increased androgen levels. This represents one monogenic adrenocortical cause of androgen excess (86).

Immunoassay for DHEAS measurement

Since DHEAS is a water-soluble antigen present in human plasma in large quantities (around 6 µmol/L in young subjects), immunoassay is performed on plasma sample with or without dilution, depending on the method

used (radioimmunoassay, automated immunoassay with a non-radioactive marker, etc.). Using highly specific antibodies renders interference from other steroids very unlikely, given the typical concentration (12).

DHEAS in hyperandrogenic states

Approximately 20–30% of hyperandrogenic and/or PCOS women show excess adrenal androgen production (AAP) when DHEAS is used as a marker of AAP (87). Hirsute patients with high DHEAS levels may show a PCOS-like phenotype. Inherited defects in the enzymes responsible for abnormal steroidogenesis account for only a very small portion of patients with increased DHEA/DHEAS. In contrast, many PCOS patients under ACTH stimulation display a generalized increase in the adrenal steroid production with no evidence of hypothalamic–pituitary axis dysfunction (88). *In vivo* studies have reported that insulin amplifies adrenal steroidogenesis in response to ACTH in PCOS (88, 89), which is consistent with *in vitro* data (90, 91). In an opposite way, metformin (92) and thiazolidinedione treatment decrease DHEAS levels in correlation with decreased insulin resistance and hyperinsulinemia in PCOS patients (93, 94). Studies in monkeys agree with these findings (95), although there are also some contrasting results in the field (96).

Regarding the influence of ovarian androgens on the secretion of adrenal androgens, the effect of testosterone is controversial in both *in vivo* and *in vitro* studies (97); in PCOS, it was concluded that ovarian androgens, and particularly testosterone, have only limited impact on adrenocortical function (87). Preliminary results from the MEDIGENE study support the concept that some genes associated with insulin resistance may be associated with excess AAP (98).

Screening for nonclassical congenital adrenal hyperplasia (NCAH) in hyperandrogenic states

Most if not all guidelines suggest that diagnosis of NCAH should be ruled out in hirsute and PCOS patients based on morning plasma 17-hydroxyprogesterone (17OHP) levels, optimally sampled during the first stage of the cycle. Basal 17OHP level should be assessed in the absence of or well after any glucocorticoid treatment that might cause false negative results. The largest multicenter trial, reported by Moran *et al*. (99), showed that baseline 17OHP level above 10 ng/mL is a sensitive criterion for NCAH diagnosis; in agreement with a more recent single-center study (100), morning baseline 17OHP <2 ng/mL was associated with 8% false negatives: i.e., patients with genetically proven NCAH. To enhance screening efficiency, an ACTH challenge test has been developed, using 250 µg cosyntropin. With a >10 ng/mL cut-off, Morel and Tardy (101) showed that nearly 100% of patients with genetically proven NCAH, regardless of the type of mutation, could be identified. This very accurate cut-off value is routinely used for NCAH screening in hyperandrogenic women (102, 103, 104, 105, 106). In the near future, the development of sequencing platforms should enable routine sequencing of the *CYP21A2* gene.

Perspectives in assessing hyperandrogenic states

The identification of androgen metabolites

Dihydrotestosterone, which is generated from testosterone by 5α-reductase, and its major glucuronidated androgen metabolites have been measured by MS (74). They could provide significant comprehensive profiles that merits to be further investigated. Interestingly, DHEAS was found to be a strong independent predictor of glucuronidated androgen in PCOS patients (74).

The identification of 11-oxygenated steroids with androgenic activity, and especially the recent characterization of 11-keto-testosterone and 11-keto-dihydrotestosterone, challenges the paradigm that testosterone is the main potent natural androgen (107). In addition, the abundance of adrenal C19 steroid 11β-hydroxyandrostenedione (11OHA4) as a precursor of 11-oxygenated steroids, and the development of ultra-performance convergence chromatography-tandem mass spectrometry (UPC2-MS/MS) for their analysis, have opened up new perspectives for understanding androgen excess disorders (108). O'Reilly *et al*. (109) elegantly showed that 11-oxygenated androgens constitute the majority of circulating androgens in women with PCOS and that obese PCOS women have higher 11OHA4 levels than non-obese PCOS women, who show close correlation between 11OHA4 and metabolic disorder markers (BMI, fasting insulin and Homa index). The direct influence effect of insulin that increases the androgen-activating enzyme aldo-ketoreductase type 1 C3 (AKR1C3) expression, together with the lipogenic effects of androgens reported in PCOS women open new fields of investigation for establishing the mutual influence of androgen excess on metabolic dysfunctions in women (110).

Main recommendations for laboratory investigation of hyperandrogenic states

Assay standardization is crucial for correct interpretation of results. Progress has been achieved through the introduction of homogeneous hormone standards. However, many automated methods for determination of steroid hormones do not provide satisfactory results. Interpretation requires knowledge of normal concentration ranges and deviations in a population of normal women free of ovarian or metabolic disorder (111, 112).

External quality assessment (EQA) of hormone assays

Since the 1980s, the trend has been for in-house assays to be superseded by automated assays, and this places a special responsibility on manufacturers to ensure reliable assay design and calibration. Since the 1980s, the trend has been for in-house assays to be superseded by commercial automated assays, and this places a special responsibility on manufacturers to ensure reliable assay design and calibration as well as for the hospital laboratories to validate the analytical performance of these assays.

Obviously, the infrastructure and management of hospitals and health care systems vary according to national policies and accreditation system. Ensuring correct implementation is a multidisciplinary responsibility involving clinicians, laboratory staff, manufacturers of diagnostic systems and healthcare regulators. All these professional groups therefore have an interest in external quality assessment (EQA). EQA is an audit tool that can identify the improvements required to reduce the risk of inaccurate hormone assessment that are of central importance for the diagnosis of endocrine disease (112). EQA has been undertaken in several countries, including France since 1977 by a Lyon-based association of biologists (ProBioQual) (13). Participating laboratories receive lyophilized control sera 6 times per year, for assay of numerous analytes, including testosterone. Analysis of results obtained over time has prompted a number of observations. The vast majority of laboratories (over 95%) carrying out total testosterone are using in routine various immunoassays without prior extraction or purification. There is considerable scatter in control serum values exhibiting concentrations close to those found in women. This variation is due to differences between assay kits and to the lack of precision of most assay kits at these concentration levels. Scatter decreases with increasing control sample concentration and becomes acceptable for concentrations comparable to those found in men (13). The limitations of testosterone assays were summarized in a position statement by the Endocrine Society (28), and a CDC Hormone Standardization Project has been promoted (113, 114, 115). Table 2 lists the pros and cons of direct testosterone immunoassay.

Decision-tree for evaluating the origin of androgen excess founded on a single total testosterone assay

Where testosterone is 2-fold higher than the upper normal limit (or >2 S.D. of the mean normal range), it is suggestive of an androgen-secreting tumor. In this case, DHEAS must be measured. DHEAS level >600 µg/dL indicates androgen-secreting adrenal carcinoma (often associated with hypercorticism). In unusual circumstances, dexamethasone test to suppress androgens arising from a functional adrenal source and gonadotropin-releasing hormone (GnRH) agonist could be helpful, in identifying ovarian androgen-secreting tumor and hyperthecosis.

Where testosterone is just above the upper normal limit, the most likely diagnosis is PCOS. However, screening for the nonclassic form of 21-hydroxylase deficiencies should be performed (17OH-progesterone assay on basal or ACTH-stimulated conditions) and, depending on the clinical setting, Cushing disease should be ruled out. Lastly, Δ4-androstenedione has been studied comparatively with testosterone, and dissociations have been reported in patients having isolated elevation of androstenedione but no elevation of testosterone particularly in case of reduced SHBG concentration that is essentially associated to metabolic syndrome.

Remarks in conclusion

Increased total testosterone level is the main indicator of hyperandrogenic states in women. By its high accuracy, mass spectrometry provides the best tool for measuring testosterone. There is debate on how to measure free testosterone, which has the advantage of taking account of the fact that SHBG, the main testosterone transport protein, is low in many circumstances, including overweight, insulin resistance and moderate inflammatory state. However, a recent review challenged the hormone-free hypothesis and its interpretation and proposed a multi-step dynamic allosteric model of testosterone's binding to SHBG assuming variability of SHBG-binding affinity and crystallography data showing that each SHBG homodimer binds two testosterone molecules rather than one (116). This concept is yet to be independently verified.

One alternative would be to measure only SHBG as a marker for these disorders, which play a part, notably in the pathophysiology of PCOS. The most exciting perspective could be to access to the complete profile of androgen precursors as well as metabolites by using performances of MS, not only in the blood but also in urine and various tissues. This future would open a new domain in the comprehension and treatment of androgen excess disorders in women.

Declaration of interest

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