

# Measuring serum testosterone in women



When requesting a blood testosterone test in women it is key to be aware of the limitations of the commercial immunoassays used by most pathology laboratories. Major limitations of reported low blood testosterone concentrations in women must be recognised before deciding whether the result is a valid, reproducible, and clinically meaningful measurement. Physiologically, blood testosterone in women derives from two sources—direct secretion by ovaries with possibly some adrenal contribution, and from pro-androgen precursors (predominantly from the adrenals in postmenopausal women) that are subsequently converted to testosterone peripherally. Women are testosterone deficient only when both adrenal and ovarian steroid production is lost, such as in panhypopituitarism.

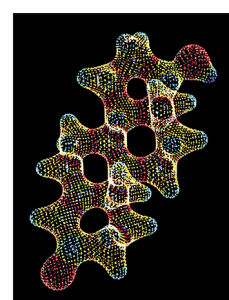
The first valid method of measuring testosterone in blood required pre-assay solvent extraction and chromatography to separate the testosterone from many structurally similar steroids and remove non-specific serum interference in the antigen-antibody reaction.<sup>1</sup> These original, laborious, manual steroid immunoassays remain valid today. By the late 1980s, simplified direct testosterone immunoassays for high-throughput, automated, multiplex immunoassay platforms were developed. Multiple key technical steps required for valid measurement were eliminated, resulting in these direct testosterone immunoassays showing method-specific bias and severe inaccuracy relative to the reference methodology of liquid chromatography-mass spectrometry (LCMS), especially in women.<sup>1</sup> At low blood testosterone concentrations in women, interpretation of the results should be restricted to whether the hormone concentration is higher than or within the reference range provided by the laboratory. Furthermore, comparing testosterone immunoassay measurements in clinical practice or between published studies might not be reliable.

Chemical mass spectrometry methods were developed at the same time as steroid immunoassays. Mass spectrometry depends on electrochemical fragmentation of the target steroids into specific molecular fragments that are quantified, with automated multiple reaction monitoring allowing for accurate measurement of the target analyte versus its reference standard, adjusting for extraction efficiency with the use of deuterated isotopic

standards. This procedure is powerfully enhanced by chromatography, an independent separation technique based on physicochemical features of the target steroid, such as polarity or hydrophobicity, employed before the mass spectrometry step. Refinements linking chromatography in tandem with mass spectrometry has culminated in modern gas chromatography mass spectrometry and, in the 1980s, in LCMS. LCMS can provide multi-analyte steroid profiles, including almost unlimited numbers of steroids from a single small (0.1–0.2 ml) serum sample without limitations of antibody specificity or serum matrix interference. In medico-legally sensitive domains, such as anti-doping and gender eligibility regulations, where detection of rule violations can preclude a professional athlete from competing, mass spectrometry-based measurements have been relied upon exclusively for decades rather than immunoassays.

Nonetheless, clinical pathology laboratories continue to use the simplified direct automated steroid immunoassays. This choice is possibly explained by lock in reagent rental agreements that provide expensive immunoassay platform equipment without cost in return for exclusive use of proprietary immunoassay reagents.

The failings and inaccuracy of testosterone immunoassays at low blood concentrations in women, together with over-reliance on, and over-interpretation of, isolated measurement of testosterone, created a desire to extract more information from the testosterone immunoassay measurements. This desire led to various derived testosterone measures, such as free or bioavailable testosterone and the free androgen index (FAI), justified by the free testosterone hypothesis.<sup>2</sup> That unproven hypothesis asserts that non-protein bound testosterone is the most biologically active fraction of circulating testosterone and therefore a better marker of androgenicity. However, this hypothesis has major conceptual and empirical pathophysiological flaws.<sup>3</sup> Conceptually, this hypothesis makes an unjustified unidirectional assertion that unbound testosterone is more biologically active when that fraction is equally accessible to sites of testosterone degradation. Hence, whether unbound testosterone is the most, or least, active moiety of circulating testosterone cannot be



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established. The same caveats apply to the so-called bioavailable testosterone.

Measuring free and bioavailable testosterone is theoretically based on the laboratory measurements of unbound or loosely albumin-bound testosterone, respectively. As these laborious, non-robust methods remain non-standardisable, surrogate formulae have been generated mostly based on equilibrium binding theory to estimate free testosterone. These binding theory formulae require concurrent measurement of serum testosterone and sex hormone binding globulin (SHBG) and are based on testosterone's potential occupation of SHBG binding sites, rather than SHBG mass.<sup>2</sup> The binding equations further disregard the four non-harmonised SHBG immunoassays available<sup>4</sup> and the genetic or disease-state variations in SHBG-testosterone binding affinity.<sup>5</sup> Empirical measurements of the binding affinity vary over at least a five-fold range,<sup>6</sup> with evidence for two different binding sites,<sup>7</sup> exerting strong influence on the calculations. Assuming a single, fixed binding affinity of testosterone to SHBG is implausible and a source of random and systematic errors. Empirically, derived testosterone measures have no certified reference standard, nor is one likely to be ascertained, as required for valid analytical variables. Without a valid standard, there is no quality control—an essential requirement for pooling or comparing results from different laboratories and creating a valid harmonised reference range. Recognising that calculated free testosterone in women's blood samples is derived from unreliable formulae, with the use of testosterone measured with imprecision by immunoassay, and variable SHBG measurements substituting for testosterone binding occupancy is key. In concert, these flawed assumptions cast serious doubt on the clinical interpretation of free testosterone in women.

Even less satisfactory is the FAI—a crude estimate of free testosterone formed by dividing testosterone by SHBG concentration. The FAI is not a reliable indicator of non-protein bound testosterone in women.<sup>8</sup> As immunoassay-measured serum testosterone in women has a narrow range (0.5–2.0 nmol/L), whereas serum SHBG has a wide range (15–100 nmol/L), the FAI is effectively a surrogate (inverse) measure of serum SHBG. SHBG, and hence the FAI, are influenced by obesity, ageing, and oral oestrogen use, without any

necessary inference for androgen action. Accordingly, FAI is closely correlated with insulin resistance (ie, with SHBG concentrations), rather than measured testosterone in postmenopausal women.<sup>9</sup>

No reference ranges for testosterone concentrations by age or menopausal status, measured with precision by LCMS, have been established in women aged 40 to 69 years. Therefore, there is no cutoff concentration below which a woman can be considered to have low testosterone. Clinicians managing women should be pressing their pathology laboratories to provide accurate testosterone measurements by LCMS, as has long been mandatory for anti-doping and gender eligibility regulations. The only indications for measurement of testosterone in women remains investigation of clinical hyperandrogenism and monitoring of pharmacological testosterone treatment.

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David J Handelsman, \*Susan R Davis  
susan.davis@monash.edu

Andrology Laboratory, ANZAC Research Institute, University of Sydney, Sydney, NSW 2130, Australia (Prof D J Handelsman MBBS, PhD); Women's Health Research Program, School of Public Health and Preventive Medicine, Monash University, Melbourne, VIC 3004, Australia (Prof S R Davis MBBS, PhD); Department of Endocrinology and Diabetes, Alfred Health, Melbourne, VIC 3004, Australia (Prof S R Davis)

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