summary and general discussion

based on ‘Testosterone assays: fitness for purpose’
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During the last decade, the quality of commonly used serum total testosterone assays has been frequently questioned [1;2]. Many of these assays have been used despite their poor performance, in the absence of viable alternatives. Data obtained from these assays may lead to faulty conclusions. The appeal of the Endocrine Society for better assays has created more awareness of this problem [3] and was taken to heart by endorsing clinical organizations, clinical laboratories performing these assays, and testosterone assay manufacturers.

**Serum total testosterone analysis**

Since we wholeheartedly agreed with the appeal of the Endocrine Society, we developed a sensitive and accurate serum total testosterone method using ID-LC-MS/MS (Chapter 2). In the analytical validation, our method showed a comparable performance to a registered reference method gas chromatography-mass spectrometry (ID-GC-MS). Moreover, we performed a clinical validation and established that our ID-LC-MS/MS method was able to distinguish testosterone concentrations between women in whom menopause was induced by removal of the ovaries and women who went into menopause naturally. Because the choice of internal standard is important, we tested two isotopically labeled internal standards in the validation process and found that these internal standards show equal performance. After this thorough analytical and clinical validation we concluded that we developed a state-of-the-art method for the reliable assessment of testosterone.

The appeal of the Endocrine Society also led to the development and marketing of 2nd generation assays by two manufacturers that show improved performance compared to their predecessors. The accuracy of the first and second generation immunoassays was investigated in Chapter 3. We used our ID-LC-MS/MS method to test the commercially available testosterone immunoassays that are most commonly used in the Netherlands, anno 2012. We found the performance of these assays in the normal male range to be acceptable. However, at low testosterone concentrations, typically found in women, there is a considerable range in quality among different immunoassays. In the immunoassay’s procedure there is a step incorporated to release testosterone from its endogenous binding protein, but its execution may not always be fully effective. We therefore also studied the effect of a manual liquid extraction before analysis. We concluded that manual sample extraction may boost the performance of most of the immunoassays, but may also lead to a less accurate calibration.

**Serum total testosterone in clinical practice**

Diagnoses of testosterone related-pathologies of patients with low testosterone concentrations, i.e. women, hypogonadal men, and children, will benefit most from the improved testosterone assays and we now have the tools to re-evaluate testosterone testing in these populations. Because of conflicting data in literature, we also
investigated the daily dynamics of testosterone parameters across the menstrual cycle in Chapter 4. Our data indicates that even though testosterone concentrations were statistically significantly elevated around ovulation, the biologic variation in individual women is independent on the menstrual cycle and of such a magnitude, that there is no need to test testosterone in a particular phase of the menstrual cycle and the reference ranges can be applied irrespective of the day of sampling. Based on the intra-individual variation we do recommend, however, that assessment of the androgen status of women is based on multiple measurements. With these data, we also re-evaluated the reference ranges for testosterone in women.

In continuation of Chapter 4, we investigated testosterone derived parameters, i.e. free testosterone and free androgen index (FAI), and the diagnostic value in polycystic ovary syndrome (PCOS) (Chapter 5). We found that the parameters that take sex hormone-binding globulin into account have a better discriminative power between healthy and hyperandrogenic women with PCOS. In addition, we conducted both studies (Chapters 4 and 5) also by a 2nd generation testosterone immunoassay. Compared to our ID-LC-MS/MS, this immunoassay showed a persistent positive bias that seems to be related to the SHBG levels. Nevertheless, there was a close analytical and clinical agreement between this immunoassay and ID-LC-MS/MS. Based this data, we were able to estimate the biological variation of testosterone in women and determine performance criteria for testosterone assays.

In hypogonadal men, either due different pathological mechanism or induced through androgen deprivation therapy in prostate cancer, testosterone concentrations are low. Especially in androgen deprivation therapy, testosterone is lowered to castration levels, i.e. < 1.7 nmol/L, and monitoring of therapy is necessary. Thus far, urologists do not request testosterone measurements consequently because this was of limited use due to poor testosterone assays [4]. With the ID-LC-MS/MS, we evaluated testosterone concentrations in patients using luteinizing hormone-releasing hormone agonist-therapy (LHRH agonist) and surgically castrated patients (Chapter 6). Remarkably, medically castrated men had lower testosterone levels than men who had their prostate removed. The clinical relevance remains unclear and should be further investigated. Also, the concentrations that we reported raise the discussion whether the cut-off value for testosterone concentrations in castrated men is appropriate or should be adjusted downwards.

The third group of patients in which an accurate serum testosterone method is required are children, this was not investigated as part of this thesis. Children are a vulnerable group and research is limited due to ethical considerations. A sample collected by a non-invasive acquisition procedure as an alternative for serum could take away these limitations. Saliva collection, for instance, is practical and essentially non-invasive. We therefore developed an ID-LC-MS/MS method to measure testosterone in saliva (Chapter 7). Apart from setting salivary testosterone reference ranges in men, we
evaluated the testosterone profile in female-to-male transgender adolescents between intra-muscular injectable testosterone esters. The extreme concentrations, i.e. supra-physiological levels after injection and subsequently relatively low levels towards the end of the inter-injection period, may affect the wellbeing of the patient. We anticipate that the method for the assessment of salivary testosterone is also applicable in children.

GENERAL DISCUSSION

**Serum total testosterone analysis**

The ID-GC-MS is considered the gold standard for serum total testosterone measurement provided it is executed properly; however, the cumbersome procedure renders this technique unsuitable for routine use. ID-LC-MS/MS is theoretically less labor intensive. In light of the still compromised performance of immunoassays, does this mean that all clinical laboratories should switch to ID-LC-MS/MS immediately?

Until now, the introduction of LC-MS/MS into clinical routine laboratories is still limited. Several reports state that ID-LC-MS/MS is a highly accurate technique for testosterone measurements. However, its accuracy strongly depends on the instrument in combination with the procedure used. If extensive sample preparation is needed in order to achieve this high performance, throughput is usually compromised to such an extent that the method is not suitable for routine use. Meanwhile, one should keep in mind that the implementation into routine practice requires financial investment and expertise, both of which aspects could be problematic, especially in small laboratories without a back-up instrument. Even though the new generation MS/MS instruments are more stable and user-friendly, downtimes of a week for troubleshooting and maintenance are not exceptional.

A great advantage of ID-LC-MS/MS is its potential to measure multiple steroids within one run. Testosterone-related disorders in newborns, i.e. ambiguous genitalia and congenital adrenal hyperplasia (CAH), are generally disorders in which the concentration of multiple steroids deviates from normal ranges. In addition, the volume of blood that can be obtained is small. Therefore, an ID-LC-MS/MS method that can determine multiple steroids in one run for routine use is particularly useful in this population [5]. The current trend towards more sensitive state of the art instruments could enable measurement of a steroid profile in saliva in the future. This could benefit these patients, however, reference values in saliva need to be established.

In summary, no fast and easy total testosterone measurement in serum with high accuracy, precision, and sensitivity is available today. It is however critical to select an assay that has sufficient discriminative power to answer the clinical question at hand, or is, in other words, ‘fit for purpose’. The performance of an assay relies on the whole
measurement procedure, so a combination of sample preparation and the analytical technique. The range of possibilities makes it obscure and difficult to select the appropriate assay. We therefore propose a categorization of the measurement procedures. With the currently available data, this thesis included, testosterone measurement procedures can roughly be divided into three categories (figure 1) – provided that the procedures are executed and validated properly – depending on the sensitivity and specificity of the procedure in relation to the reference ranges for different patient groups and the application of different measurement procedures in common clinical settings in which testosterone assessment is required.

A measurement procedure may include sample preparation steps such as liquid extraction or protein precipitation to release testosterone from its endogenous binding protein and clean up the sample. Category 1 consists of assays with acceptable performance to measure high serum total testosterone concentrations, typically found in healthy males, while category 3 on the other end consists of procedures with comparable performance to the reference method GC-MS. In an ideal world, testosterone would only be measured by a method with comparable performance to an established reference method. Even though great efforts are being made by the parties involved to standardize testosterone assays [6-8], poorly performing assays that are not suitable to answer all clinical questions are still in use. In order to interpret testosterone concentrations properly, it is crucial to realize the limitations of the method used in relation to the reference ranges for different groups of healthy men and women. In
figure 1, the different measurement procedures are categorized and the average functional limit of quantification, which takes the sensitivity and specificity into account, is displayed in relation to the reference ranges in figure 2. With this information, an overview of the clinical applicability of the different categories of assays has been generated as shown in figure 3.

Testosterone concentrations should always be interpreted relative to the specific reference intervals of the individual clinical laboratory. Taking into account the wide intra-individual variation, testosterone measurement on more than one occasion is preferable. Other factors that could influence testosterone concentrations should also be taken into account, such as lifestyle, physical and mental wellbeing, body mass index, pregnancy, testosterone altering medication, i.e. androgen deprivation therapy, hormonal anti-conception, DHEAS supplements, and anabolic supplements.

FIGURE 2: Male and female serum total testosterone reference ranges with the mean functional limit of quantification of different assay categories. The reference ranges are derived from publications using accurate thoroughly validated measurement procedures. Reference ranges are laboratory specific and considering the broad range in quality of different methods, the depicted reference ranges should only be used as guidelines [9-12]. The functional limit of quantification is a rough estimate of the mean and may vary among individual measurement procedures and laboratories.

**Serum total testosterone in clinical practice**

*Adult male*

Physiological testosterone concentrations in males are relatively high and therefore quite easy to estimate accurately by current commercially available methods [13]. Initially, any assay is sufficient to differentiate between either normal (high) or hypogonadal (low) testosterone concentrations (see figure 3). Calculated free or bio-available testosterone
may help to confirm diagnosis when borderline concentrations (7–12.5 nmol/L) are encountered [14;15]. Which testosterone measure is more suitable for what diagnosis in men has been extensively reviewed by Diver [16]. A category 1 assay will not suffice for prostate cancer patients receiving androgen deprivation therapy. To monitor the current therapeutic cut-off value of < 1.7 nmol/L, at least a category 2 assay is needed.

**FIGURE 3**: Applicability of different assay categories in clinical practice.

**Adult female**

Testosterone-related pathologic conditions in women can be divided into two groups: hypoandrogenism and hyperandrogenism. Hypoandrogenism in women is associated with a decreased libido and quality of life. Its diagnosis, however, is controversial because of the limitations of testosterone assays [17]. In the last decade, much attention has been paid to this issue, resulting in more awareness and the development of more accurate and sensitive methods (category 3), which will facilitate further research in this area. Increased testosterone concentrations (hyperandrogenism) are encountered in polycystic ovarian syndrome (PCOS) and with hirsutism, acne, excessive adrenal steroid synthesis, infertility and virilization, oligo-/amenorrhea, and non-classical congenital adrenal hyperplasia (NCCAH). A category 2 assay should be sufficient to discriminate between normal and increased testosterone concentrations. If the testosterone concentration does not match the clinical presentation or if testosterone concentrations are borderline increased, the measurements are best repeated using a category 3 assay (see figure 3). In a review by Kane and co-workers [18] an overview of the measurements that can be performed to confirm a diagnosis of testosterone-related pathology is presented.
**Pediatric**

In pediatrics, testosterone is mainly assessed to confirm disorders of puberty or pubertal stage. Concentrations may vary from ‘undetectable’ to the normal adult male range (figure 2). A category 2 assay is preferred for this population with a more sensitive and accurate category 3 assay as backup. Obtaining a large sample volume from this vulnerable group of patients is not desirable, making GC-MS analysis usually not suitable, which of course also holds for neonates.

**Neonate**

In the first week of life, a range of steroids is present in the circulation at high concentrations [19]. Using direct immunoassays for testosterone measurements is almost useless during this period because they will give such high testosterone results due to cross-reactivity that an androgen-producing tumor cannot be excluded from the differential diagnosis. After the first week, although some prefer after the first month, an accurate immunoassay (category 2) could suffice. However, a diagnosis is usually urgent and cannot always wait until after the first week. For research purposes, e.g. into the programming of the hypothalamus-pituitary-gonadal axis in premature infants, a category 3 assay is indicated.

**Androgen-producing tumor**

Androgen-producing tumors are very rare but should be mentioned as they can occur in all patient groups. A highly increased testosterone level could indicate the presence of an androgen-producing tumor. However, hyperandrogenic disorders are mostly a combination of increased androgens, making cross-reaction in immunoassay also very likely. A specific category 3 assay may exclude possible analytical errors. In addition, multiple steroid-analysis might facilitate the identification of the tumor and the cells involved.

**Other measures of testosterone**

Serum total testosterone is the most frequently measured androgen in clinical practice. However, according to the free hormone hypothesis, free testosterone concentrations are more reflective of the physiologic actions as only free testosterone is able to diffuse into the target tissue to bind to the androgen receptor. The analytical limitations described above for serum total testosterone also apply to free testosterone measurements, possibly even more because free testosterone concentrations are only 1-3% of total testosterone.

In general, there are three methods to determine free testosterone. First, the gold standard for measuring free testosterone which includes ultrafiltration or dialysis as sample preparation followed by a highly accurate testosterone assay such as GC-MS or
LC-MS/MS [20]. This method is seldom performed in clinical practice for several reasons: the first rationale is that not all diagnostic laboratories have access to mass spectrometry and secondly separation of the free testosterone fraction is time consuming. The third motivation is that one may also question the validity of free testosterone measurements because of several analytical challenges: separation of the free fraction in serum may inevitably lead to a new equilibrium and even slight fluctuations in temperature, pH, buffer, and dilutions may significantly alter the equilibrium and hence the estimated concentration of free testosterone [21]. Any deviation from physiological conditions should be avoided which is practically impossible. During dialysis, serum is dialyzed against a buffer, separated from the sample by a membrane, which in essence results in a dilution of the sample. The alternative for dialysis could be ultrafiltration. The principle of ultrafiltration seems relatively straightforward: serum in an ultrafiltration unit is centrifuged, the unbound testosterone fraction passes through the filter, and the bound testosterone remains behind. The analytical challenge in this procedure is the risk of adsorption of free testosterone to the filter. If one has succeeded to separate the free testosterone from the protein bound testosterone, there is the analytical challenge of measuring the concentration of the separated free testosterone. The free fraction (dialysate or ultrafiltrate) plus the bound fraction (residu) should be equal to the total testosterone concentration in a sample. However, the most accurate testosterone assay (GC-MS) has an analytical variation (±3%) that is greater than the free fraction of testosterone in serum (±2%). To correct for procedural losses, an analogue or isotopically labeled internal standard is added. However, addition prior to the separation process may alter the free and bound testosterone equilibrium, whereas addition after the separation process does not take the possible losses during dialysis or ultrafiltration into account.

Taking the fragility of the testosterone equilibrium into account, it would be preferable to validate against an established reference method. A thoroughly validated candidate reference method for free testosterone by ultrafiltration has been published; however, it uses filters that are no longer available [20]. Chen and co-workers [22] describe a method using a different filter, but one might argue that some aspects of their validation procedure are questionable. In addition, the amount of adsorption to the filter was not tested and the method was not validated against a registered reference method.

The second option to estimate free testosterone is the most commonly used method: the calculation of free testosterone using concentrations of total testosterone and its binding proteins. However, the accuracy and precision of the calculated results depends on the accuracy and precision of the assays used for total testosterone, SHBG, and albumin, and the algorithms used. Sometimes a fixed average albumin concentration is used, obviously with associated inaccuracy. The accuracy of different algorithms has been evaluated [23-25]. The third option to estimate free testosterone is
by direct immunoassays that are commercially available, though, these assays seem to be very inaccurate and the least reliable option [26-28].

Since only free testosterone is able to diffuse into saliva, it has been proposed that salivary testosterone concentrations reflect serum free testosterone. The analysis of testosterone in saliva has never become popular, mostly because commercially available assays lack sensitivity, specificity and reproducibility [29]. However, the use of saliva potentially has great advantages: sample collection is minimally invasive and saliva is a practical alternative when sequential measurements of serum testosterone are indicated. Sample collection may take place at any time of the day as no professional is needed and samples are stable to be transported mailed to the laboratory. In theory, saliva is ideal for sampling with short-term dynamic testing protocols. Because of these advantages and the introduction of sensitive ID-LC-MS/MS testosterone methods, it is attractive to explore the options of salivary testosterone in clinical practice. As testosterone is generally assessed in serum, future research should also look into the relation between serum testosterone and salivary testosterone and may include the evaluation of reference ranges for salivary testosterone at different ages throughout childhood. The introduction of less invasive sample collection may also enable us to research the hypothalamus-pituitary-adrenal axis activation in newborns.

Tissue
As described above, it is currently possible to assess testosterone in serum and saliva. Measuring testosterone in any of these matrices is only a substitute for the actual testosterone status, as testosterone diffuses into target tissue to exert its biological activity, either after conversion to the more potent androgen dihydrotestosterone or not. Testosterone and dihydrotestosterone can diffuse into cells where it binds the androgen receptor and regulates transcription [30]. The measurement of tissue testosterone concentrations is limited to research settings. However, the technological developments have improved the sensitivity of testosterone measurements. The measurement of testosterone and other androgens in prostate cancer tissue for example may enable us to investigate the effects of androgen deprivation therapy in the target tissue and possibly offer personalized therapy.

Conclusion
Testosterone assays have improved significantly since the appeal of the Endocrine Society for better assays. Progress has been made, but the final goal is not reached yet. Due to financial considerations, laboratories are often forced to compromise in quality of testosterone measurements. Nevertheless, great efforts are being made – and should continue to be made – to arrive at a situation in which testosterone values are comparable across methods, time, and location. All parties are encouraged to pay close attention to the validation of assays, including the pre-analytical phase. A technique on
its own, i.e. GC-MS or LC-MS/MS, is not a gold standard: the sensitivity and accuracy of a method is based on the protocol being used. In addition, the interpretation of testosterone values, including values that are determined by a gold standard, should be done carefully taking biological variation and laboratory specific reference ranges into account.

REFERENCES


