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Chapter 8

Testosterone Measurement and Prostate Cancer

Tine Hajdinjak

Additional information is available at the end of the chapter

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1. Introduction

Testosterone is important growth factor for prostate cells. If testosterone availability drops, prostate cells stop thriving. Benign prostate shrinks and the same happens with prostate cancer cells. Larger decrease in testosterone availability means larger reduction in prostate cells mass. Although only reduction in testosterone levels will not, in most occasions, permanently heal prostate cancer, it causes its regression and significantly delays further progression of prostate cancer. Therefore, reduction of body's testosterone level is important prostate cancer treatment modality. When surgical removal of prostate due to cancer is not an opinion (for example because of advanced age, significant comorbidity or because cancer has already spread beyond prostate) or was unsuccessful as noted by rising PSA, which indicates cancer growth, serum testosterone value becomes very important factor in treatment related decisions. If testosterone values are high, reduction of testosterone level will be helpful – it is expected prostate cells will react, shrink, PSA will fall. If testosterone values are already low, their further reduction with different agent may be possible. If testosterone values are already at the lowest reachable levels, other ways of treatment should be sought. After reduction of testosterone levels in the body (castration), prostate cancer cells with time (sometimes months, sometimes years, sometimes decades) develop alternative signaling mechanisms and ways of paracrine androgens supply. It is estimated this happens in a third of all prostate cancer patients [1].

As this chapter focuses primarily on prostate cancer, some topics, like free-testosterone or salivary testosterone measurements are not included, because although they are related to testosterone measurement in general, they are, at least at present (things may change in the future), not used in day-to-day care of prostate cancer patients. All testosterone values mentioned relate to serum testosterone measurements.

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2. Some characteristics of testosterone

Testosterone is principal male androgen, sex hormone and anabolic steroid. It is found not only in humans, but also in many other vertebrates. In males, testosterone is secreted by Leydig cells in testicles, in females by theca cells in ovaries. Small amount is produced also in zona reticularis of adrenal cortex in both genders and in placenta. Chemically (figure 1), it is white powder, soluble in methanol, name is17beta-Hydroxyandrost-4-en-3-on or 4-Androsten-17beta-ol-3-on, Chemical Abstracts Service number 58-22-0, ATC code G03BA03. It is a controlled substance, in US by Drug Enforcement Administration (DEA). It's inactive epimer – difference in configuration of OH at C17 - is called epitestosterone. Testosterone's biosynthesis starts from cholesterol. Metabolism: up to one tenth of testosterone is converted by 5-alpha reductase to dihydrotestosterone, less than 0.5% by aromatase to estradiol. Most of testosterone is deactivated and excreted as glucoronides.

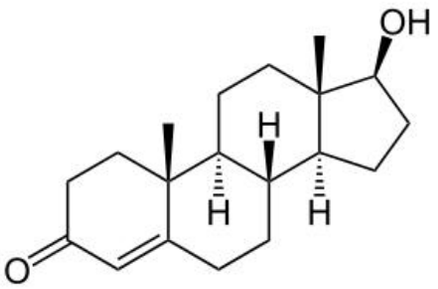


Figure 1. Testosterone structure (Picture in public domain – Wikimedia: NEUROtiker)

3. Reasons for testosterone measurement in prostate cancer

Testosterone measurement in prostate cancer patients has more than 40 years history [2]. Confirmation of castrate testosterone level is necessary before identifying prostate cancer as castration resistant. Castrate states are at present defined as serum testosterone level below 20 ng/dl (=0.69 nmol/l) or below 50 ng/dl (=1.73 nmol/l) [3], but it was not always this way and different testosterone measurement methods have important implications.

Need for controlling quality of chemical castration treatment of prostate cancer steams from reports of up to 15% castration failures [4,5]. This means LHRH treated patients may not reach castration levels of testosterone due to different reasons [6], not only non-compliance, application failures, but also other reasons, for example problems with depot formulation resorption due to granuloma formation on injection site [7] or may simply need more frequent dosages [8].

Further reason for testosterone measurements in prostate cancer patients lies in reports of correlation between success of castration and time to PSA progression: better castration

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(lower testosterone value) gives longer time to progression [9,10]. Therefore hormonal treatment of prostate cancer should not be followed with PSA measurement only (as indirect indication of treatment success), but also with testosterone measurement [11].

Before any treatment, at diagnosis, serum testosterone value is predictor of disease aggressiveness – lower testosterone values are related to less differentiated cancer and worse prognosis [12]. For all stated reasons, measurement of serum testosterone is important for clinicians who treat prostate cancer patients.

After long term of androgen suppression with LHRH (GnRH) analogues, sometimes testosterone levels do not recover after stopping treatment (which may be due to permanent dysfunction of Lydig cells), therefore application of LHRH drugs may be stopped in selected patients [13]. However, this should be confirmed and followed with testosterone measurement.

But testosterone measurements are not important only for urologists, who, apart from main reason – decisions related to prostate cancer management, use it for example also for aging male symptomatology and evaluation of patients with erectile dysfunction. Also other medical specialties, like endocrinology, pediatrics, gynecology or oncology use testosterone measurements for their conditions, like diagnosing and monitoring hyper- or hypo- androgenic disorders in women, like polycystic ovary syndrome, alopecia, acne, hirsutism or hypoactive sexual desire disorder; androgen secreting neoplasms; congenital syndromes with ambiguous genitalia... Pediatrics and endocrinology were in the past probably most frequent users of testosterone assays, but nowadays most laboratories receive most testosterone requests from urologists.

4. Prostate cancer incidence will increase in future

Prostate cancer is already most frequently diagnosed cancer among men in the developed world. As a cause of death among males, it is second in the USA and third in Europe. Large increase in prostate cancer incidence in recent years is not only due to availability of PSA (biochemical marker, which is useful for screening purposes) and due to better awareness of doctors and population at large, but in large part also due to changes in population pyramid and increased life expectancy. As breast cancer, which is most common in females over 60 years of age, also prostate cancer is cancer of older people. For example, in Slovenia (which may be in health related issues regarded somewhere in-between developed western and less advanced other parts of the world), incidence of prostate cancer increased 50% from 2000 to 2011 [14]. At the same time, population at main risk (males above age 60) increased 28%. Therefore more than half of increase of prostate cancer incidence can not be attributed to, as some people, even health care professionals, claim, "artificial" increase of incidence due to "over-screening", but simply to the fact that population at risk has significantly increased. And among those (males between 55 and 70), screening is most appropriate because life expectancy also increases (at present, for 75 year old man in Slovenia it is on average more than 10 years) and therefore cancer control is worthwhile.

In our country, recently prostate cancer incidence has been higher compared to breast cancer. Cause for this is not better prostate cancer "screening", but simple fact of changes in population pyramid, in numbers of populations at risk: relation between males and females in most important age range for prostate and breast cancer detection has changed – number of males grows significantly faster than number of females. In year 2000, 700 more females reached age of 60 compared to males, in 2011, 500 more males reached age 60 compared to females [15]. Although among oldest old, number of females will remain higher compared to men, present big gap in number of men compared to women in age group 50-70 is getting smaller and smaller and this also contributes to further increase of significance of prostate compared to breast cancer.

According to population pyramid, further increase of burden due to prostate cancer is expected, for example in our country, until year 2050, when overall population in Slovenia will, according to present trends, decrease from current 2 to 1.9 million, but number of males, age 60 or more, will peak at 1.8 times the number in 2011. Similar trend is expected to happen in most countries in the world sooner or later and therefore prostate cancer will remain important health problem in future.

5. Need for hormonal treatment of prostate cancer may not decrease in future

Despite facts about prostate cancer incidence, presented in section 4 and despite undeniable proof that population based PSA prostate cancer screening reduces mortality due to prostate cancer [16], it seems some professional bodies, like U.S. preventive services task force [17,18] recently advised against screening.

Further, among young UK general practitioners, during non-formal conversation, in year 2012, one can easily hear claims like "PSA – oh I thought it is NOT for screening, it is only for follow up purposes, only for patients, who have diagnosis of prostate cancer already" (personal experience).

With this recent trend by policy-makers, it seems hopes of urologists, who treat prostate cancer patients, that we will in the future find only very few patients, who will present with stage of disease, where nothing else but hormonal treatment would be possible or hormonal treatment will become necessary during the course of their disease, are dispelled. As it seems focus of attention is turned away from early detection and managing (watchful waiting, not necessary treating patients with prostate cancer), towards second and third line treatments for advanced disease, testosterone measurement in patients with prostate cancer will become even more important in the future.

6. Different hormonal treatments influence testosterone differently

Different drugs for hormonal treatment of prostate cancer have different effects on serum testosterone. Non-steroidal antiandrogens increase overall serum testosterone levels. Steroi-

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dal antiandrogen (cyproterone) reduces testosterone levels, but not to castrate values. Often old patients take two 100 mg tablets daily and testosterone values are than commonly around 7 nmol/l. With proper dosing (3 times 100 mg daily), values nearing castration levels have been reported (mean 2.5 nmol/l, [19]), on the other side, with dose 200 mg daily, relatively small decrease only to low-normal levels has been reported for healthy young to middle-aged men (mean 11.4 nmol/l [20]).

LHRH agonists injections are supposed to universally reduce testosterone levels to castration values, but sometimes this is not the case. LHRH antagonists are gaining popularity very slowly with similar effect on testosterone. They may reduce testosterone levels in a proportion of patients a bit further compared to LHRH agonists [21] and they do not cause microsurges of testosterone, which are often present with every re-dosing of LHRH agonists.

Surgical castration remains a viable opinion in many countries and for many patients. Steroids are available to further reduce serum androgen levels in castrate resistant disease states by blocking adrenal production. 5 alpha reductase inhibitors may, according to some theories, play a role in combination treatment.

In the past, castrate values of testosterone were achieved with estrogens, like stilbestrol. Due to side effects (blood cloths), this is not used any more. Ketoconazole, inhibitor of steroid synthesis, is still available for fast testosterone levels reduction, but in practice is is used mainly in experimental settings after chemotherapy failure in castration resistant states [22].

Typical testosterone responses to some hormonal agents are summarized in Table 1.

Agent	Typical testosterone response
non-steroidal antiandrogen (bicalutamide, flutamide)	increase (may go above 30 nmol/l)
steroidal antiandrogen (cyproterone acetate)	decrease, very dependent on dosage regimen, with 3x100 mg it may approach, but not reach castrate values, in a few days
GnRH (LHRH) agonists (triptorelin, goserelin, leuporolide)	designed to decrease levels below castrate values (below 1.73 nmol/l), may take a month after first application to reach castrate level
GnRH antagonists (degarelix)	designed to decrease levels below castrate values without surges
surgical castration (bilateral orchiectomy)	gold standard, decrease below castration level in few hours, however, adrenal androgens remain
ketoconazole	decrease below castration levels if dose is high enough in 2-4 days, but sometimes variable response, corticosteroids should be supplemented simultaneously
estrogens (stilbestrol – of historical interest only)	decrease below castration levels after approx. 5 days, later surges may appear

Table 1. Typical serum testosterone responses to different hormonal agents. In practice, individual responses may vary significantly, therefore confirmation with individual measurement is important.

7. Methods for serum testosterone measurement

With introduction of indirect RIA techniques (double isotope derivative dilution technique) to measure serum testosterone in 1970ties and later automated chemiluminescent assays, serum testosterone values became widely available to practicing urologists.

Manufacturers mainly use similar principles of assays. As an example of principle, Abbott's chemiluminescent assay is described [23]. It is "delayed one step", competitive heterogeneous assay. First, testosterone in serum sample is displaced from sex binding globulin (SHBG) with low-pH buffer. Sample is mixed with microparticles, coated with mouse monoclonal anti-testosterone antibody. After incubation, addition of labeled testosterone (in this case, conjugated with alkaline phosphatase), follows. Labeled testosterone binds to unoccupied sites on microparticles, coated with the antibodies against testosterone. More testosterone in the sample – less sites are free for labeled testosterone to bind. After another incubation, reaction mixture is transferred to cells, where microparticles fix and bind. Wash step follows – it removes unbound conjugate (labeled testosterone and other substances which may interfere with next step). Then, labeled antigen is visualized and measured. Signal is inversely proportional to amount of testosterone in the sample – as according to principle of competitive assay – stronger signal indicates more added, with marker conjugated testosterone present, therefore less "original" testosterone in the sample. In Abbott's example, 4-methylumbelliferyl phosphate is added and alkaline phosphatase, conjugated to added testosterone, hydrolyzes phosphate from 4-methylumbelliferryl phosphate to 4-methylumbelliferone, which fluorescence is measured [23].

In direct RIA methods, principle is the same, only marking of competing antigen is performed with radioactive substance instead of alkaline phosphatase or other enzymatic, fluorescence-based technique. Large variability was observed for direct RIA methods [24]. In indirect RIA methods, quantification follows organic solvent extraction and purification steps with monitoring of procedural losses. Although correlations between indirect RIA and mass spectrometry methods are good (above 0.9), absolute concentrations were reported to be significantly higher, probably (as in direct assays), due to cross-reaction of immunoreactive material [25].

Indirect assays (extraction and chromatography followed by RIA) are not available any more in our practice. Main method for serum testosterone determination in most present day clinical laboratories around the world (perhaps it is different in parts of US) is still direct automated chemiluminescent assay [26]. This assay mixes antibodies directly with serum and skips extraction step. This holds true for all direct assays, not only chemiluminescent but also radio-immuno (RIA) based.

Mass spectrometry (MS) of steroid compounds, which includes testosterone, has a long history of research and development [27]. It is coupled to liquid chromatography (LC, a separation technique in which the mobile phase is liquid) or gas chromatography (GC, a separation technique where the mobile phase is gas). After first separation and before ionization, in the past, derivatization (conversion of chemical compound into derivative) was often used to

improve, for example, ionization efficiency and other characteristics of analyte[28]. With development of more sensitive techniques, today derivatization seem not included any more in a typical setting for testosterone determination with HPLC-tandem mass spectrometry. Sample must be ionized before ions are separated according to mass and charge in the spectrometer. Among methods of ionization are for example atmospheric pressure photoionization (suggested to be most optimal for testosterone analysis) or (less optimal for testosterone) electrospray ionization. Tandem mass spectrometry (MS/MS) means that spectrometry is performed in an arrangement in which ions are subjected to two or more sequential stages of analysis (which may be separated spatially or temporally).

High throughput LC/MS/MS has become gold standard for measurement of testosterone and other well defined steroid substances in biological fluids. GC/MS can also be used to quantify testosterone, but represents today mainly a "discovery tool" which provides "integrated picture of individual's metabolome" [29].

Some characteristics of testosterone assays are summarized in Table 2.

Type	Characteristics
chemiluminescet	uses antibodies, direct, most laboratory platforms (Abbott, Siemens, Roche) have their own antibodies, which all cross react to some extent to other substances and give consistent, but different results, typically higher than reference methods in/near castrate range
RIA – radio -immuo assay	uses antibodies, rarely in use those days, typically good results if indirect – radio- immuno - detection after chromatography step, for direct RIA's, same as for chemiluminescence – problems with antibody selectivity
LC-MS/MS: liquid chromatography – tandem mass spectrometry	uses molecular mass based identification, indirect, uses different liquid chromatography methods to extract testosterone from sample (for example "high turbulent flow") and tandem mass spectrometry to confirm and quantify sample, gold standard
GC-MS: gas chromatography – mass spectrometry	uses molecular mass based identification, indirect, research mainly, useful for profiling different steroids in the sample, reference method, issues with "in-house" development, sample preparation, most labor and resource intensive

Table 2. Most prevalent types of testosterone assays.

8. Units for testosterone measurement

Guidelines [3] state testosterone values in ng/dl only and some countries still use old values (for example US, Germany, Belgium), but in many countries laboratory results only in SI units - International System of Units - (nmol/l) - are available (for example Slovenia). Some articles, to further confusion, use other combinations, like ng/ml or mg/dl. To allow easier reference to practicing physicians, in Table 3, some typical serum testosterone values are presented in different units.

Conversion factors: as molecular formula of testosterone is $C_{19}H_{28}O_{2,}$ molecular mass of testosterone is 288.42 g/mol. Therefore, if value in ng/dl is available, multiply it with 0.0347 nmol/l / ng/dl to get value in nmol/l. If value in nmol/l is available and one needs ng/dl, value in nmol/l should be multiplied by 28.8 ng/dl / nmol/l to get ng/dl. 1 ng/ml (or microg/l) = 100 ng/dl.

Clinical meaning	value
normal morning value for males, above	12 nmol/l (= 346 ng/dl = 3.46 ng/ml)
advised supplementation for healthy males, regardless of symptoms, below	8 nmol/l (= 231 ng/dl = 2.31 ng/ml)
"old" castration value	1.73 nmol/l (= 50 ng/dl = 0.5 ng/ml)
median value for premenopausal females	1.39 nmol/l (= 40 ng/dl = 0.4 ng/ml)
"Morote's" value	1.11 nmol/l (= 32 ng/dl = 0.32 ng/ml)
"new" castration value	0.69 nmol/l (= 20 ng/dl = 0.2 ng/ml)

Table 3. Typical serum testosterone values in different units. "Morote's" value represents level of serum testosterone, determined with direct chemiluminescent immuno assay in prostate cancer patients on hormonal treatment, above which shorter time to progression was observed compared to patients with testosterone values below this level [9]. For curiosity, median value for premenopausal females can also be used as guideline for supplementation in hypoactive sexual desire disorder [30].

9. Daily rhythm of testosterone

Circadian and "ultradian" mean testosterone level fluctuations peak is around 8 AM and through level around 8 PM. Over this, there is a 90 min oscillation in testosterone values as reflection of pulsatile secretory pattern.

Sleeping increases testosterone values [31]. Some even claim sleep, not circadian rhythm to be more important for regulation of testosterone [31]. Pattern of physical activity (physical work or training in the morning versus evening) does not influence testosterone concentrations or testosterone diurnal pattern [32]. Food (mixed meal) decreases testosterone value, if blood is taken 1-2 hours after, by 30% in comparison to overnight fast [33]. Better sleep increases testosterone value [34]. Anxiety may increase testosterone levels, it was even suggested, patient's samples on the day of admission to hospital should not be used because anxiety may be associated with increased testosterone level [35]. On LHRH agonists, diurnal pattern is expected to be abolished [36]. Age reduces circadian fluctuations [37].

Due to stated variations in testosterone levels during the day, morning fasting blood samples are standard.

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10. What can one expect from direct chemiluminescent assays –Example

Wide availability of automated testosterone assays should make easy for clinicians to follow prostate cancer patients testosterone levels, as at present almost every clinical laboratory offers testosterone measurement with one of direct chemiluminescent assays methods.

Aim was to evaluate use of such a testosterone measurement tool in every-day clinical practice and consequences that might follow. Claims from some pharmaceutical company representatives on their LHRH agonist formulations to be better than others were also addressed.

10.1. Materials and methods

In a cross-sectional audit study, serum testosterone level was determined in all patients on 3-month LHRH formulations, treated in out-patient clinic in two months period. Blood samples were taken immediately before the next injection. Only patients, who previously received more than one injection and with previous injection exactly 3 months or less before examination were eligible.

Three preparations were found to be used: Diphereline (triptorelin 11.25 mg), Eligard (modern leuprolide formulation, 22.5 mg) and Zoladex (goserelin 10.8 mg).

Further 10 samples were taken from patients with surgical castration performed more than 6 months ago, who appeared on regular follow up out-patient visit during the study period.

Testosterone measurement was performed with direct chemiluminescent microparticle immunoassay Architect from Abbott Laboratories. According to procedural leaflet, functional sensitivity of this assay was 0.49 nmol/l (95% confidence interval 0.38 – 0.59) and analytical sensitivity 0.28 nmol/l.

As SI units (nmol/L) are obligatory in our country, all testosterone measurements were originally reported in SI units and conversion to US units (ng/dl) was performed for the purpose of this report using conversion factor of 0.0347.

For statistical evaluation of differences between groups of patients on different LHRH agonist formulations, analysis of variance between groups was calculated using open source statistical software R [38].

10.2. Results

125 patients aged 50 to 92 (median 74 years, lower quartile 70, upper quartile 78 years) were included.

For the whole group, serum testosterone values ranged from 14 ng/dl (0.5 nmol/l, lowest reportable result) to 107 ng/dl (3.7 nmol/l), median 37 ng/dl (1.3 nmol/l), lower quartile 32 ng/dl (1.1 nmol/l), upper quartile 58 ng/dl (2.0 nmol/l).

According to those results, considering castrate level of 20 ng/dl (=0.694 nmol/l), only 7% of patients on LHRH treatment and 2/10 patients after surgical castration could be classified to

castrate state of disease. Considering castrate level of 50 ng/dl (1.735 nmol/l), 66% of patients on chemical castration and 8/10 patients after surgical castration would comply.

Testosterone measurement results, according to LHRH agonist, are presented in Table 4. According to analysis of variance, differences between groups of patients, treated with different LHRH agonists, were not significantly different (F=0.69, p=0.5).

LHRH formulation	N	TST:min-max	TST-median	TST-75%	TST-90%
triptorelin 11.25 mg	53	20-98	37	58	72
goserelin 10.8 mg	41	14-107	37	52	69
leuprolide 22.5 mg	21	14-84	49	63	72

Table 4. Testosterone measurement results with Abbot Architect assay in patients on different 3-month LHRH agonists. Samples were taken immediately before next injection. TST – testosterone. Units: ng/dl (1,73nmol/L=50 ng/dl). Differences between different LHRH formulations were not statistically significant.

11. Problems with direct testosterone immunoassays

Large differences were reported from measurements of the same serum sample with chemiluminescent assays from different manufacturers [39,40]. Direct RIA techniques were not better [41]. In the low range (values of interest for castration control in patients with prostate cancer), which was close to range of female testosterone levels, direct assays gave results more than 20% different from the gold standard [41]. Abbot Architect assay was also reported to give consistently up to 20% higher results compared to standard in this range of values [39].

One of the reasons for variability is in the fact that antibodies are different among manufacturers, with different cross-reactivity profiles. All present direct chemiluminescent assays are matrix dependent, which was extensively studied by the British group [42]. It was confirmed there was significant cross-reactivity for example with dehydroepiandrosteronesulphate (DHEA-S) [43]. The described issue is not only in urology regarding testosterone – also other areas of endocrinology where steroid hormones measurements are important, have reported and discussed similar issues [44,45]. College of American Pathologists proficiency testing revealed in 2008, highest mean compared to lowest mean for testosterone, to differ by factor 2.8 [46]. Differences for mass spectrometry assays were much lower, by factor 1.4.

12. Problems with mass spectrometry testosterone assays

Mass spectrometry (MS) assays are not commercially available in classical sense, but are to much larger extent dependent on each laboratory's own development. As mass spectrome-

try technology is capable of very high sensitivity and specificity, those assay are accepted as gold standard. But, they are more than direct commercial assays dependent on proper calibration and sample preparation[47]. Research has shown biases as high as 25.3% for testosterone values near castrate ranges [47]. Others reported up to 26% of results outside total error limit of 14% due to improper calibration and between-run calibration [48]. Although MS techniques are becoming standard assays for steroid hormones, this presents several challenges, for example affordability for smaller laboratories, high operating costs of equipment, need for standardization of MS assays and in many occasions, actually setting new reference ranges [49] and relating them to physiological and pathological conditions, as happens with testosterone, where castrate values have been moved from 50 ng/dl to 20 ng/dl.

13. Castrate testosterone values in different prostate cancer studies

Serum testosterone value around 1.735 nmol/l or 50 ng/dl as castrate level for the purpose of hormonal treatment of prostate cancer was used already in 1970'ties [2]. Later, some LHRH formulations were designed to achieve serum testosterone below this value in 95% of treated patients. It was accepted as standard value in guidelines [50]. Guidelines have at present gone even a step further and stated testosterone levels above 50 ng/dl to be in-sufficient and additional hormonal manipulation to be warranted in such patients [3]. It is further generally accepted patients with surgical castration to have lower levels of testosterone – around 15 ng/dl and certainly below 30 ng/dl [51]. As surgical castration provides lower testosterone levels, there were always claims one should aim as low as possible with testosterone levels and should try to reach below 20 ng/dl – for example in a small study of 38 patients, treated with LHRH agonists, Oefelein found 5% did not reach values below 50 ng/dl and 13% did not reach values below 20 ng/dl [52]. This movement, which aims to decrease castrate testosterone level, was further supported by publication which claims patients with castrate testosterone levels below 32 ng/dl (1.1 nmol/l) – Morote's value - to have longer time to biochemical progression [9]. In their study, which also used chemiluminescent antibody testosterone assay, in 25% of patients testosterone levels above 50 ng/dl were identified. Further, with serial measurements, 55% of patients on chemical castration had testosterone values found above 20 ng/dl [8]. Studies which use HPLC/MS/MS for determination of testosterone levels do see lower values [53].

Some studies seem to oversee guidelines and post their own castrate testosterone levels, which are significantly higher and set to a value which offers approximately 95% successful castration. In their article on testosterone escape, group from Norway claims their castration level is 2.8 nmol/l which equals 81 ng/dl [6]. This value was selected as their laboratory's upper normal limit for women. And with this value, they identified 10% of patients who failed to reach this castration level. The present study was similar to this in testing patient's serum for testosterone at the end of 3 month dosing interval, which may also influence results.

Another group from Turkey, which evaluated influence of androgen deprivation therapy on hand function in 2008 article used radioimmunoassay for testosterone measurement and in a

castrate group mean value of testosterone was 52 ng/dl +- 35 ng/dl [54]. One can assume for approximately half of their patients testosterone levels were not in castrate area according to guidelines. Surgical castration study, using chemiluminescent assay, found values up to or above 50 ng/dl for surgically castrated patients [55]. Further surgical castration study found patients on LHRH treatment before surgical castration to have values above 50 ng/dl in 28% of patients and after surgical castration in up to 8% [56]. Unfortunately method of testosterone measurement is not stated in this article, but it correlates perfectly with data presented here, where chemiluminescent method was used. Further, recent LHRH agonists report from Canada, which also used "competitive immunoassay using direct chemiluminescent technology" [57], found median testosterone values for different LHRH agonists to be (in nmol/l) 1.2, 1.3, 1.1 and 1.3 and in two of five formulations, upper quartal value was 1.8, indicating 25% of patients on particular formulation to be even above "old" castration value of 1.72 nmol/l (50 ng/dl). Another study from Canada, also using chemiluminescent immunoassays, although claiming they were "newer technology", indicates risk for breakthrough levels of serum testosterone (value measured higher than castrate value) in patients on LHRH agonist injections to be 5.4% and 2.2% (for castration values 1.1 nmol/l and 1.7 nmol/l, respectively) per each LHRH injection [58]! Cancer control was claimed to be inferior in patients with breakthroughs of serum testosterone measured [58].

14. Direct testosterone assays and prostate cancer – The verdict

Probably one of most important reasons for observed discrepancies in testosterone measurements lies in "matrix" issue, in cross-reactivity. Immunolite assay and Abbot Architect both cross-react with DHEA and give consistently higher values for serum testosterone in range of castration male values [39,42]. Therefore results of studies, which use direct chemiluminescent testosterone assays in clinical setting cannot be compared to studies, which use chromatography followed by mass spectrometry techniques, because they do not measure the same things.

Inaccuracy of present day direct testosterone assays is already recognized in the field of female and male testosterone replacement, in pediatrics [59] and should be recognized also in the field of prostate cancer. Until indirect testosterone assays applying mass spectroscopy become widely available, publications should set realistic values of castrate levels and precisely state measurement methods used. They may be universally available in the USA, but in Europe, even western university hospitals are not quick in replacing direct immuno-assays with gas chromatography methods – for example in Ghent they changed only recently, also for reasons like "one can not publish any more anything about testosterone without this method". And even mass spectrometry methods show significant errors and inconsistencies.

On the downside, it becomes clear using direct present day techniques to control castration methods (either chemical or surgical) is not appropriate and invariably leads to disputable results. Above findings also in part explain long term debate about subcapsular or classical simple orchiectomy and part of an occasional finding of non-castrate testosterone level after

orchiectomy [56]. Also our own impulse for studying the field come from initial observations that patients after surgical castration have higher testosterone values compared to guideline's requests.

On the upside, direct chemiluminescent assays do measure something. They can unmask occasional testosterone outlier (skipped dose of drug, granuloma formation or an individual in need for more frequent dose of a drug – reduced dose interval, as explained for example in dr. Garnick's editorial comment [8]). They can identify hypogonadal men with prostate cancer before starting androgen deprivation therapy, who have very bad prognosis or may in the future benefit from modified treatments, like incorporating early use of new antiandrogens (for example MDV3100 [60]). They are necessary if one embarks on "on demand" redosing of LHRH agonists [61].

It is obvious chemiluminescent direct testosterone measurements do not show only testosterone values and as such can not serve as a tool to decide which LHRH agonist reduces testosterone more compared to other drugs. But results of such assays, as for example Abbot Architect testosterone assay, are consistent [39] and according to published and our results, there are great differences in measured levels of androgens in patients on LHRH agonist therapy (740%, from 0.5 to 3.7 nmol/L, 14 – 107 ng/dL). Perhaps, at present a pure speculation, chemiluminescent assays, which give consistent results, only with some cross-reactivity and therefore systematic overestimation of testosterone values in the low range, like Architect and Immunolite, can give estimation of overall serum androgen levels. Importance of extratesticular androgens is becoming more and more evident [62,63]. This may explain findings from Morote et al, who used same technically problematic direct chemiluminiscent assay and found correlation between assay results and time to biochemical progression [9] or from Perachino et al, who found even correlation between assay results and survival [10]. Also Hashimoto et al [64], although failing to provide details about their testosterone assay and reporting questionably low testosterone values, report usefulness of testosterone measurement for prediction of antiandrogen treatment results – when testosterone levels were low, no additional clinical benefit of antiandrogen treatment was observed, when testosterone was higher, antiandrogens were useful. If future can confirm those propositions, direct testosterone tests, despite their imprecision for their original purpose, may well serve us in selecting patients for antiandrogen addition to castration or for secondary hormonal treatment, especially in perspective of new androgen manipulating drugs, like abiraterone acetate (Zytiga) and MDV3100 [60].

15. Conclusions

Serum testosterone levels provide objectivity for proper prostate cancer disease states characterization. Testosterone level before treatment may add to prognosis. More importantly, testosterone levels during treatment become main issue in individual's prostate cancer treatment decisions, as soon as increasing PSA levels indicate failure of primary local treatment.

Apparent difference between guidelines (which ask for 20ng/dl) and practice in serum testosterone values of hormonally treated prostate cancer patients was investigated and could be explained in methodologies of testosterone determination. Most present day available testosterone assays in hospitals are direct assays, which overestimate testosterone values in the castrate range. Antibodies cross-react with other androgens in serum (which prevail in low testosterone range) and result is overall androgen estimation, not pure testosterone value. Studies should recognize this and find use for this "overall androgen" value, which is, contrary to indirect mass spectroscopy assays, universally available and was found to be related to disease progression and treatment results. Further, it is useful for identification of high risk patients with low testosterone values at diagnosis and identification of patients with poor response to LHRH agonists. Testosteron results are necessary for prolongation of interval between injections, which may be possible in approximately half of patients on LHRH agonists treatment where values are well below castration levels and at the same time, some patients may need injections of LHRH agonists in shorter intervals. In the future, tests which estimate not only pure testosterone, but overall androgen level, may become clinically relevant with awareness of prostate cancer cell's ability to use different androgen molecules and as a consequence patient tailored use of new androgen manipulating drugs.

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Author details

Tine Hajdinjak[1,2,3]

Address all correspondence to: tine.hajdinjak@gmail.com

1 Center UROL Maribor, Slovenia

2 Medical Faculty, University of Maribor, Maribor, Slovenia

3 Division of Urology, Department of Surgery, General Hospital Murska Sobota, Slovenia

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