Blood-based LC-MS/MS analysis of steroids in prostate and ovarian cancer diagnostics

The importance of adequate bioanalytical methods

Bloed gebaseerde LC-MS/MS analyse van steroïden in de prostaat- en ovariumcarcinoom diagnostiek

Het belang van adequate bioanalytische methoden

(met een samenvatting in het Nederlands)

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de Rector Magnificus prof. dr. H.R.B.M. Kummeling
ten overstaan van een door het college voor promoties ingestelde commissie,
in het openbaar te verdedigen op donderdag 30 maart 2023 des ochtends te 10.15 uur

door

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Geboren op 10 december 1989 te Rotterdam

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The research described in this thesis was conducted at the department of Laboratory Medicine of the Netherlands Cancer Institute – Antoni van Leeuwenhoek Hospital in Amsterdam, the Netherlands. Financial support was received from the Netherlands Cancer Institute to print this thesis.

ISBN: [INVULLEN]

Lay-out: Lennart van Winden
Cover: Lennart van Winden, [NAMEN]
Printing: [PRINTER]

Cover. [BESCHRIJVING COVER]

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Preface

Testosterone and prostate cancer

Worldwide, prostate cancer is the most common non cutaneous cancer among males and an important cause of mortality (1). In recent decades, significant improvements in patient survival have been achieved, primarily due to effective detection and treatment of early-stage prostate cancer (2, 3). Advanced prostate cancer, however, is still an incurable disease. Since the discovery of androgen dependence of prostate carcinoma in 1941 (4), androgen deprivation therapy (ADT) is considered to be the backbone of metastasized prostate cancer treatment. The efficacy of ADT is monitored by periodical analysis of prostate-specific antigen (PSA). When prostate cancer progression under ADT occurs despite adequate androgen deprivation, as defined by circulating testosterone concentrations of < 1.7 nmol/L, patients are diagnosed with castration-resistant prostate cancer (CRPC) (5-10). At this clinical stage, several additional options have become available in the recent years as next-in-line treatment to increase patient survival (7). In recent years, several additional androgen suppressive agents and hormonal drugs have become available. These agents focus on either lowering testosterone levels or inhibiting cellular androgen signaling (11, 12). Although these treatments increase advanced prostate cancer survival, development of treatment resistant prostate cancer is inevitable.

Testosterone analysis in the medical laboratory

To quantitate the low testosterone levels that occur in castrated men, various assays are available to medical laboratories. Testosterone is mainly measured using automated immunoassay (AIA) technology, the advantages of which include random access and short assay and turnaround times (13). However, such immunoassays tend to lack sensitivity and specificity in the sub-nanomolar and picomolar concentration range (14-16). To overcome the limitations associated with AIA, there has recently been a shift towards routine application of an LC-MS/MS-based analysis of steroid hormones, including testosterone (17-19). Notably, this technique is currently considered to be the best practice for steroid analysis and is recommended for pediatric and female testosterone analysis. Furthermore, prominent scientific journals require that testosterone analysis for primary study endpoints be performed using LC-MS/MS (18, 20). In the case of testosterone, strong clinically relevant deviations in quantitation between AIA and LC-MS/MS have been observed in women (21, 22) and neonates (23). However, as determined by a recent systematic review, for castrated prostate-cancer patients investigations on the necessity of using LC-MS/MS-based testosterone assays are lacking and, to our knowledge, have not yet been published (24).

Clinical applications of testosterone

It still remains to be determined how the improved analytical sensitivity of the LC-MS/MS-based testosterone assay can be used to support the clinical management of various stages of prostate cancer and thus benefit patients. Further clinical research is required to establish whether such low testosterone concentrations are clinically significant and whether this new method can support treatment decision-making and can be valuable as a prognostic or predictive marker. Notably, previous studies have found indications for sensitive testosterone quantitation as a prognostic biomarker of ADT efficacy. In a post-hoc analysis of the phase III COU-AA-301 clinical trial, a relationship between baseline testosterone levels and overall survival was found in CRPC patients treated with abiraterone and ADT. Herein, higher testosterone baseline levels were associated with shorter times to death compared to lower baseline levels (25). Another notable study in CRPC patients treated with abiraterone and ADT showed that testosterone, and other androgens, correlated with progression-free survival (PFS), while prominent molecular markers, such as PTEN loss and MYC amplification showed no association with PFS (26). Other studies also describe similar relationships, for example in CRPC patients treated with enzalutamide and ADT, but evidence is limited due to low sample size or application of AIA for the quantitation of testosterone (27-29).

Sex steroids, sexual functioning and the menopausal transition

Testosterone and other sex steroids, such as androstenedione, estradiol (E2) and estrone (E1), have also been associated with sexual functioning and menopausal complaints in postmenopausal women. Previous studies showed that hormonal replacement therapy (HRT, eg. Testosterone or E2 supplementation with sex steroids) can alleviate some of these symptoms in postmenopausal women and oophorectomized premenopausal women (30-34). However, other researchers investigating endogenous sex steroid levels and sexual functioning in postmenopausal women did not find a link (35, 36). In addition to these contradictory findings, these studies mostly apply AIA for the quantitation of low circulating sex steroids found in these women. While it remains difficult to investigate the relationship between biochemical data and questionnaire-based symptoms, new approaches in study design and application of accurate and reliable analytical methods could provide a better insight in the precise role of sex steroids in the menopausal transition.

Thesis outline

Although blood-based quantitation of sex steroids has been performed for over 50 years, differences in quality of various assay techniques are not always recognized in clinical

research. Furthermore, using adequate LC-MS/MS assays, currently regarded as the gold standard, could provide a better insight into the relationship between biochemical data and clinical information. This thesis describes the development and technical validation of LC-MS/MS assays, the state-of-the-art, for the measurement of several sex steroids. Applying these assays, this thesis aims to 1) reveal discrepancies between a testosterone LC-MS/MS assay and commonly applied AIA in castrated prostate cancer patients, 2) study the association between on-treatment testosterone levels and survival in advanced PCa and 3) investigate the relationship between circulating sex steroids and menopausal complaints in women undergoing oophorectomy. Hopefully, the emphasis on the importance of adequate analytical methods in this thesis contributes to the understanding among a broader audience. Furthermore, I hope that this work will ultimately aid in the implementation of testosterone as an independent risk factor for advanced prostate cancer patients and aids in the improvement of menopausal complaint mitigation.

Section 1 describes the development and validation of bioanalytical LC-MS/MS methods for the quantitation of sex steroids in human serum. Validation was based on the guidelines published by the Clinical & Laboratory Standars Institute (37). In Chapter 1.1, the importance of detailing the analytical method in clinical studies is underlined. Chapter 1.2, 1.3 and 1.5 describe the method development and validation of LC-MS/MS-based testosterone and estrogen assays. Chapter 1.4 highlights an openly available R-script for the estimation of biological variation that was used in Chapter 3.

In **Section 2**, the testosterone LC-MS/MS assay is clinically validated in prostate cancer patients. First, Chapter 2.1 provides a general overview is provided of testosterone analysis in the context of prostate cancer, while Chapter 2.2 demonstrates the differences between four automated IA and one testosterone LC-MS/MS assay in castrated prostate cancer patients. In Chapter 2.3 and 2.4, the predictive value of testosterone in HSPC and CRPC patients is explored, respectively.

Finally, **Section 3** discusses another clinical application of serum sex steroid LC-MS/MS assays. In Chapter 3.1, associations between longitudinal changes in sex steroids, menopausal complaints and sexual functioning are investigated.

Although their complexity, long hands-on time and assay time, LC-MS/MS assays have a technical advantage over automated AIA. In this thesis, 1) the validation of blood-based LC-MS/MS assays for the quantitation of various sex steroids is described, 2) substantial discrepancies between commonly applied sex steroid IA and their LC-MS/MS assay counterparts were revealed in relevant study populations, 3) during treatment testosterone levels were associated with progression-free survival and 4) changes in sex steroid levels

correlated with menopausal complaints and sexual functioning in women undergoing oophorectomy. This thesis concludes that castrate levels of testosterone and postmenopausal levels of E2 should, at least considering technical aspects, be measured by an appropriately sensitive and accurate LC-MS/MS assay. Furthermore, the results reported in this thesis warrant further investigation into the application of testosterone as an independent risk factor in advanced prostate cancer and suggest that testosterone supplementation might aid in the mitigation of menopausal complaints in postmenopausal women.

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Section 1: Development and validation of LC-MS/MS methods for the quantitation of steroids in serum

Chapter 1.1

Letter to the editor:

Reporting the analytical method is essential to assessing studies in which biomarkers are a major study objective

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Reinisch *et al.* (1) recently studied the efficacy of endocrine therapy in treating breast cancer in men. Their primary objective was to compare changes in estradiol concentrations within three treatment arms; their secondary objectives included measuring changes of concentration of several hormonal biomarkers. Although we greatly appreciate the authors' efforts to perform this study, we were surprised and disappointed by the absence of any information on the analytical methods used to measure the hormone concentrations. The method used to quantify any biomarker, and especially hormones, can significantly affect study outcome and replication (2). Analytical methods may differ in accuracy of the obtained test results, sensitivity and specificity for the biomarker, analytical traceability, and standardization required for translation to other methods (2). Therefore, adequate reporting of the employed analytical method is essential if the obtained results are to be properly assessed and replicated.

Analysis of low testosterone and estradiol concentrations, as observed in castrated men, is highly challenging. The immunoassay-based estradiol tests generally applied by medical laboratories lack the required lower limit of quantitation (LLOQ), accuracy and correlation with the liquid chromatography mass spectrometry-based (LC-MS) reference method (3, 4). For estradiol, a significant systematic error of approximately 50% and individual sample errors of up to 149% have recently been described in men for the estradiol assay marketed by Roche Diagnostics (4). The limit of detection (LOD) for the estradiol assay of 5 ng/L reported in Reinisch *et. al.* is identical to the LOD of the Roche assay, which might indicate the use of this inferior method. It should be noted that, to ensure reliable numerical test results, it is more appropriate to use the LLOQ than the LOD. For the Roche assay, the LLOQ is five times higher (25 ng/L) and still permits a 30% error. The aspects referred to in this paragraph illustrate how important it is to know which analytical method was used to measure estradiol concentrations in the Reinisch *et. al.* study.

For reporting diagnostic accuracy studies, the STARD checklist has become available to enhance reproducibility of such studies, including reporting of the analytical method (5). We strongly recommend that, also for other types of study in which biomarkers are a major study objective, adequate reporting of the applied analytical method should be recognised as essential. This reporting should at least include specification of the method and the manufacturer, as well as traceability to relevant technical performance specifications (2).

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Chapter 1.2

Serum testosterone by liquid chromatography tandem mass spectrometry for routine clinical diagnostics

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Abstract

In clinical diagnostics, low testosterone containing samples cannot be analyzed by random access immunoassays normally available at clinical laboratories. For these samples sensitive and specific LC-MS/MS based testosterone methods are required. Here, we describe a LC-MS/MS based testosterone assay developed and validated for routine clinical application.

Keywords: Testosterone, LC-MS/MS, steroids, hormone

1. Introduction

Testosterone is the most important male sex hormone (androgen) in terms of potency and amounts secreted (1). In clinical diagnostics, circulating testosterone concentrations are used for diagnosis and follow-up of various diseases such as hypogonadism, polycystic ovary syndrome and precocious or delayed puberty (2). Furthermore, in prostate cancer, androgen deprivation therapy aimed at suppressing testosterone, is the most successful first line treatment for advanced prostate cancer (3). Monitoring proper androgen suppression and identification of resistance to androgen deprivation therapy requires measurement of circulating testosterone concentrations.

In clinical laboratories, analysis of testosterone is performed mainly by automated, random access immunoassays. The advantages of these routine assays are their high throughput, low turn-around time properties. Furthermore, they do not require specialized machinery (HPLC and mass spectrometer systems) and expertise (3). However, for low concentrations of testosterone as observed in pediatric, female and castrated male samples, these immunoassays lack sensitivity and specificity (3). For such samples, application of liquid chromatography-tandem mass spectrometry (LC-MS/MS) based testosterone assays is required for accurate testosterone analysis. In addition, recognizing the limitations of the testosterone immunoassays, leading journals now require testosterone analysis by LC-MS when testosterone is used as a major end-point in studies (4). Our hospital serves a relatively large population of prostate cancer patients.
For routine application of testosterone analysis by LC-MS/MS in clinical diagnostics we developed and validated a liquid chromatography tandem mass spectrometry-based testosterone assay. Next to the analytical characteristics, the practical aspects of minimization of the hands-on time and minimization of potential errors, were taken into account when the assay was designed.

2. Materials

2.1 Stock solutions

1. Preparation of testosterone stock solution: Dissolve 10 mg testosterone (Sigma Aldrich, St. Louis, MO, United States) in 3.473 mL DMSO to a concentration of approximately 10 mmol/L.
2. Preparation of internal standard (IS) working solution: Dissolve 10 mg d5-testosterone (CDN isotopes, Pointe-Claire, QC, Canada) in 3.473 mL DMSO to a concentration of approximately 10 mmol/L.

2.2 Calibration curve

1. For a concentration of 10 µmol/L, 10 µL stock solution is mixed with 9990 µL methanol. Subsequently, final testosterone concentrations of 1, 3.3, 10, 33, 100, 333 and 1000 nmol/L are established by serial dilution in methanol (See Note 1 and Note 2).
2. The calibration curve is standardized against the NIST standard reference material (SRM) 971 (See Note 3).

2.3. Other reagents and materials

All solutions prepared in this method are of analytical grade or better and ultrapure water (purified deionized water, with a sensitivity of 15 MΩ-cm at 25 °C) was used.

1. In total, 3 serum-based control levels are used. The quality control (QC) samples are made from left-over patient materials and pools are aimed at levels representing (1) (chemically) castrated men, (2) female normal range and (3) adult male concentrations.
2. Storage: Patient serum samples are stored for up to 1 week at 4 °C before analysis. QC samples are stored long term at – 20 °C (See Note 4).
3. Preparation of system suitability test (SST) working solution: For a concentration of 10 µmol/L, 10 µL stock solution is mixed with 9990 µL methanol. Subsequently, a final testosterone concentration of 5 nmol/L is established by dilution.
4. Extraction solvent: Methyl tert-butyl ether (MTBE) is used to extract steroids from human serum.
5. Injection solution: Methanol and water are mixed to a 7:3 ratio (See note 5).

2.4. Liquid chromatography tandem mass spectrometry system

1. HPLC system: Liquid chromatography was performed on a Dionex Ultimate 3000 HPLC system consisting of a vacuum degasser, pump, and an autosampler. The autosampler needle can pierce the cap of plastic microvials (See Note 6) and has an injection volume range of 1 – 100 µL.
2. Mobile phase: The mobile phase is composed of water (containing 0.1% formic acid) (hereafter referred to as mobile phase A) and methanol (hereafter referred to as mobile phase B).
3. Separation column: reversed-phase C-18 column (5 µm particle size, 2.1 x 100m internal diameter, Agilent Zorbax Extend C-18)(See Note 7).
4. Ion source: Turbo V electrospray ionization(ESI) (AB Sciex, Concord, ON, Canada).
5. Mass spectrometer: API4000 (AB Sciex, Concord, ON, Canada) triple quadrupole mass spectrometer

3. Methods

3.1 Preparation of samples and controls

1. Add 250 µL human serum to a 2 mL safelock microvial (See note 8 and 9).
2. Add 25 µL calibration standard (See Note 10) in 1.5 mL tubes.
3. Add 100 µL SST working solution in 1.5 mL tubes.
4. Add 10 µL IS working solution to calibrators, blanks and serum samples. The SST, calibrator, blank and double blank samples are excluded from step 4 to 8 (See Note 11).
5. Add 1 mL of extraction solvent, MTBE, to the serum (See Note 12 and 13).
6. Mix for 15 minutes on an orbital shaker (See Note 14 and 15).
7. Centrifuge at 3000 g for 5 minutes at room temperature (RT).
8. Snap freeze the human serum using dry ice and ethanol (See Note 13 and 16).
9. Decant the extraction solvent into a 1.5 mL soft cap micro-tube (See Note 6).
10. Dry all samples in a speedvac concentrator in combination with a vapor trap (See Note 17).
11. Reconstitute the sample extract in 100 µL injection solution.
12. Mix (400 rpm) for 10 minutes at RT (See Note 18).
13. Centrifuge the samples at 18213 g for 5 minutes at RT (See Note 19).

3.2 Liquid chromatography

1. Purge the system with 50% mobile phase A and 50% mobile phase B at 1mL/min (See Note 20).
2. Equilibrate the column at least 10 minutes prior to run with 20% mobile phase A and 80% mobile phase B (See Note 21).
3. Apply a run time of 5 minutes with an isocratic mobile phase flow.
4. Set the injection volume to 50 µL.
5. Program an autosampler wash cycle between each sample to minimize cross-contamination.

3.3. Tandem mass spectrometry settings

1. Set ESI settings according to the listed values in Table 1.
2. Measure in multiple reaction monitoring (MRM) mode. Select the quantifier and qualifier transitions for testosterone and testosterone-d5 (See Table 2).
3. Tune for optimal MRM settings. The obtained optimal MRM settings for the collision energy (CE), declustering potential (DP), entrance potential (EP) and cell exit potential (CXP) for both the mass transitions of our system are listed in table 2 (See Note 22).
4. Set the dwell time to 100 ms.

3.4. LC-MS run and batch design

1. The analytical run starts with three system suitability tests (SST) containing 2 nmol/L testosterone standard and IS (See Note 23).
2. Next, 8 calibrators including a blank are measured in duplicate.
3. Double blank samples in duplicate are introduced after the calibrators (See Note 24).
4. Two sets of QC samples (3 levels, singular samples) are used; one set placed before and one set placed after the patient samples. (See Note 25 and 26).
5. Patient samples are scheduled in between the sets of QC samples and run in duplicate. (See Note 27).

3.5. Data analysis

1. Starting data analysis, chromatography of the quantifier and qualifier transitions are checked for their similarity. Aberrant peak shapes between quantifier and qualifier transitions, such as shouldering or twin peaks, are an indicator of interference (See Note 28).
2. Next, in quantification mode, integration of peaks is automatically performed and manually reviewed and corrected when inaccurate automated integration was observed. Integration of the analyte should match integration of the corresponding IS (See Note 29).
3. For calibration, the testosterone / IS ratio is calculated using the quantifier transition.
4. A calibration curve is generated using a linear regression and a $1/x^2$ weighting is applied (Note 3 and 30).
5. Patient and QC results are individually calculated using the calibration curve.
6. QCs are individually reviewed and a 2SD control rule is applied for every control.
7. For patient samples the duplicate results obtained are averaged and the average concentrations is reported as testosterone concentration.
8. The difference between the duplicate sample results relative to the mean concentration was calculated and used to control for sample handling errors (See Note 31).

4. Notes

1. The calibration curve range is 0.1 – 100 nmol/L. By using a concentrated calibrator stock the standard volumes added to the calibrator samples can be decreased accordingly. This method prolongs the usage of one set of standard stock.
2. Generally, a calibration curve is made in the same matrix as the QC and patient samples. However, a human serum pool with undetectable levels of testosterone is difficult to obtain in the required volumes. For this reason, we verified whether the

methanol calibration matrix was suitable as an alternative to the serum matrix and chose to use methanol as calibration matrix.

3. The assay was standardized against the serum based NIST (National Institute of Standards and Technology) reference material SRM 971. The two reference material samples were analyzed in 4 independent runs and both SRM 971 standards were diluted in various concentrations to confirm trueness throughout the measuring range.
4. Serum testosterone stability was tested as part of the method validation. Testosterone stability in collected serum was at least 1 week when stored at room temperature, at least 2 weeks when stored at 4 °C and at least 2 months when stored at – 20 °C. Furthermore when serum was not separated from the separator gel containing collection tube after centrifugation, testosterone was stable for at least 1 week when stored at 4 °C (5, 6). Stability was defined as a ≤ ±6% bias from the fresh testosterone sample.
5. Injection solution can be stored at RT. However, to avoid contamination we prepared injection solution on a weekly basis.
6. Specifically, we used Brand soft cap micro-tubes (Wertheim, Germany) in combination with the cap piercing of the autosampler. Therefore, no certified glass sample vials are needed.
7. Our assay was validated with a Agilent Zorbax Extend C-18 column, since this column is also used for other applications run on the LC-MS/MS system.
8. A 250 µL sample size was chosen as this offers a sufficiently low lower limit of quantification (LLOQ) of 0.17 nmol/L with acceptable sample consumption. At this level a total CV of 5.88% and a mean signal-to-noise ratio (S/N) of 14 are obtained. The application of higher-end mass spectrometers (2, 7) or incorporation of derivatization methods (8, 9), allow higher assay sensitivity, though derivatization processes are often more time-consuming and therefore not preferred for routine clinical diagnostics.
9. For this step, we used 2 mL Safe-lock Eppendorf tubes (Hamburg, Germany). Safe-lock tubes provide additional protection against spilling during the mixing step. We find that normal tubes often lack cap security resulting in sample loss.
10. Note that by reconstitution of sample extract of 250 µL serum in 100 µL injection solution the samples are concentrated 2.5 times. This is accounted for by adding a volume of 25 µL standard working solution into the calibrator samples.
11. SST, calibrator, blank and double blank samples do not contain serum. Therefore, no extraction is needed for these samples.
12. MTBE is highly volatile. This results in inaccurate pipetting using air displacement pipettes. We find that using a repeater pipet increases accuracy of distributing equal volumes significantly.
13. Precautionary measures are accounted for by working in a fume hood.

14. As a reference, we performed sample mixing with an IKA LS 130 basic orbital shaker (IKA, Staufen im Breisgau, Germany).
15. We find that by placing the sample vials sideways in the shaker, the mixing is performed with improved efficiency. By increasing the surface area the immiscible liquids distribute more equally inside the tube.
16. For snap freezing a Styrofoam container is used. First, a permeable micro-tube rack is placed in the container. Subsequently, dry-ice is placed around the rack. Ethanol is then poured into the container. Importantly, the ethanol should cover the aqueous phase in the micro-tubes. Freezing the aqueous phase usually takes 20 – 40 seconds, depending on the volume of ethanol and the amount of dry ice. A good indicator is the forming of a projection on the surface of the frozen serum.
17. As a reference, we used a Savant SC210A Speedvac Concentrator (Thermo Fisher Scientific, Waltham, MA, United States) in combination with Refrigerated Vapor Trap RVT4104 (Thermo Fisher Scientific, Waltham, MA, United States) to dry the organic phase after extraction.
18. The samples should not be mixed sideways as this step focuses on reconstitution of the dried sample, which is mainly positioned at the bottom of the tube.
19. By centrifuging the samples shortly, droplets of dissolved sample located in the upper part of the tube are repositioned at the bottom of the tube. Additionally, in the case of increased turbidity in the samples, which is often present in human serum, this step acts as purification of the sample. After intensive centrifugation, the turbidity extract is pelleted at the bottom of the tube. As a result, the turbidity extract remains in the tube during injection of the sample.
20. Generally, the system is purged for at least one minute. This results in clearance of any present air bubbles in the tubing leading to the pumps.
21. Testosterone and the corresponding IS have a tested retention time around 2.1 minutes using a 80:20 mobile phase B:A ratio in our system set-up (See Figure 1).
22. Details on the general settings and MRM settings of testosterone and testosterone-d5 used in our method are displayed in table 1 and 2 respectively. Note that the parameters of the IS corresponding to the analyte are identical. Firstly, variations in parameter values between analyte and IS could result in signal differences troubling quantification. Secondly, replacement of hydrogens by deuterium yield a molecule that has virtually identical chemical-physical properties. Therefore, tuning both molecules should result in similar values for all parameters.
23. An SST is performed in triplicate, prior to the analytical run to confirm proper system functioning before starting an analytical run.

24. Normally, double blank samples are used to check the matrix and LC-MS/MS system for contaminants and interferences. In this case, however, the calibration curve is generated in methanol and double blank samples are introduced to identify a possible contamination in the IS working solution or the injection solution.
25. The QC samples serve as a measure of the quality for each run. When a significant aberration of QC concentration is detected (exceeding a 2SD rule), the run and obtained results require additional evaluation before obtained results can be released.
26. Assay precision was tested by measuring 3 QC levels in quadruplicate for 10 individual runs. Total coefficient of variation (CV) was 4.7, 4.1 and 3.3% for testosterone concentrations of 0.4, 3.1 and 7.6 nmol/L respectively.
27. We find that increasing the number of samples reduces practicality of the sample pretreatment. Therefore, we recommend to divide large patient serum sample sets (i.e. > 20 patient serum samples) over multiple runs.
28. No interference was detected from DHT, androstenedione, DHEA, 17-OHP, cortisol, hemolysate, bilirubin and intralipid. Only epitestosterone tested positive for interference. However, to date no epitestosterone interference (i.e. a peak at a retention time of 2.4 minutes) has been observed in any patient sample analyzed.
29. Generally, the Analyst® software package (Version 1.6.2) integrates peaks automatically based on pre-determined settings. In general these settings should provide correct integration in more than 90% of samples, but at low concentrations (smaller peaks) fluctuation may result in poor automatic recognition of peak start and ending. Correct where needed and ascertain peaks of the analyte and the corresponding IS are integrated similarly.
30. The applied $1/x^2$ weighting was selected based on overall trueness and linearity results obtained during assay validation. We determined that a $1/x^2$ weighting outperformed a 1/x weighting assay performance, especially for lower testosterone concentrations that we considered most relevant. Recently, a similar observation was reported (2).
31. Patient serum samples are measured in duplicate to detect laboratory handling errors. In the case of inconsistent results (difference of duplicate results> 15%), the sample should be considered for re-analysis.

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Table 1. General settings of the ESI ionization source.

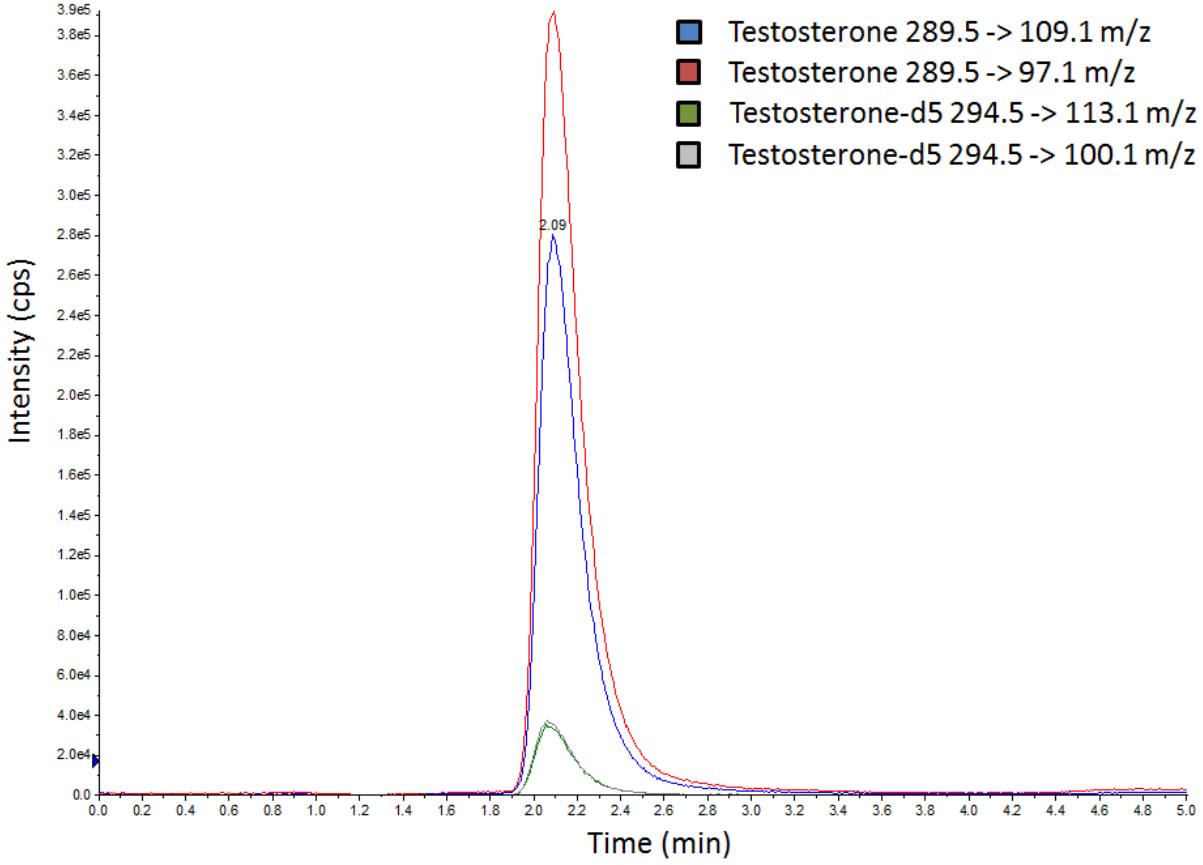
General settings	
Nebulizer gas (psi)	50
Curtain gas (psi)	20
Ion spray (V)	5500
ESI temp (°C)	500

Table 2. MRM settings.

		MRM Transition (m/z)						
Quantifier		***Q1***	***Q3***	***CE (V)***	***DP (V)***	***EP (V)***	***CXP (V)***	***Dwell time (ms)***
	Testosterone	289.5	109.1	35	90	10	7	100
	Testosterone-d5	294.5	113.1	35	90	10	7	
Qualifier		***Q1***	***Q3***	***CE (V)***	***DP (V)***	***EP (V)***	***CXP (V)***	***Dwell time (ms)***
	Testosterone	289.5	97.1	35	110	10	7	100
	Testosterone-d5	294.5	100.1	35	110	10	7	

Figure 1. Testosterone chromatogram

Chromatogram of a calibrator sample containing 25.4 nmol/L testosterone. The sample is spiked with 2 nmol/L IS. Blue represents the quantifier testosterone transition, red represents the qualifier testosterone transition, green represents the quantifier testosterone IS transition and grey represents the qualifier testosterone IS transition.



Chapter 1.3

Retrospective analysis of serum testosterone levels by LC-MS/MS in chemically castrated prostate cancer patients: Biological variation and analytical performance specifications

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Abbreviations:

17-OHP	17-hydroxyprogesterone
ADT	androgen deprivation therapy
APS	analytical performance specifications
B	bias
CAS	chemical castration only
CI	confidence interval
CRPC	castration-resistant prostate cancer
CV_A	analytical variation
CV_I	within-patient variation
CV_G	between-patient variation
DHEA	dehydroepiandrosterone
ENZA	chemical castration with enzalutamide
IA	immunoassay
II	index of individuality
IS	internal standard
LC-MS/MS	liquid chromatography tandem –mass spectrometry
LLOQ	lower limit of quantitation
NIST	National Institute of Standards and Technology
RCV	reference change value
PSA	prostate-specific antigen
TE	total error
QC	quality control

Abstract

Background: A sensitive liquid chromatography tandem-mass spectrometry (LC-MS/MS) method was used to monitor serum testosterone levels in castrated prostate cancer patients. We subsequently performed an observational and retrospective study to estimate the within- and between-subject biological variation of these patients.

Methods: In total, 474 samples from 72 prostate cancer patients in the Netherlands receiving either chemical castration (CAS) or castration plus enzalutamide (ENZA) treatment were selected for data analysis. ANOVA was performed to estimate analytical variation (CV_A) and within-patient variation (CV_I). A nested ANOVA was applied to estimate between-patient variation (CV_G). From these data, the reference change value (RCV) and analytical performance specifications (APS) were calculated.

Results: Testosterone levels were significantly higher in the ENZA group (0.318 vs. 0.191 nmol/L, $p<0.005$) than the CAS group. Overall, variation components were estimated at 6.1%, 24.6% and 60.3% for CV_A, CV_I and CV_G, respectively. Both groups showed high individuality (<0.6). The RCV was 70.3% for all patients. Desirable APS were 12.3% for imprecision, 16.3% for bias and 26.4% for total error.

Conclusion: The generated APS are valuable for sensitive testosterone assays and the high individuality indicates that castrated testosterone levels can be studied as a predictive or prognostic biomarker in prostate cancer patients.

Key words: LC-MS/MS, steroids, testosterone, androgen deprivation therapy, prostate cancer, biological variation

Introduction

Serum testosterone levels are clinically applied in chemically castrated prostate cancer patients as a monitoring marker for adequate castration and treatment. Currently, these patients are predominantly treated with gonadotropin-releasing hormone agonists and are diagnosed with castration-resistant prostate cancer when the cancer progresses under adequate androgen deprivation (1). In recent years, several additional therapeutics targeting the androgen signaling pathway have become available. Among other effects, these agents focus on inhibiting cellular androgen signaling (Enzalutamide) (2-6).

Based on 50 year old double-isotope derivative assays, adequate androgen deprivation is defined by circulating testosterone concentrations of < 1.7 nmol/L (7-9). Notably, recent studies applying chemiluminescent immunoassays (IA) indicate that a cut-off at 0.69 nmol/L is more appropriate (10-13). While these assays have obvious advantages for clinical laboratories, they tend to lack sensitivity and specificity necessary to accurately quantitate testosterone in low concentration ranges (14-16). Furthermore, desirable assay performance specifications (APS), estimated from biological variation data in these patients, are currently lacking to safeguard adequate testosterone monitoring in this setting. Notably, with the development of the more sensitive and accurate liquid chromatography tandem-mass spectrometry (LC-MS/MS) assays, biological variation studies investigating testosterone dynamics in castrated prostate cancer patients are now enabled.

Previous biological variation studies investigating serum testosterone are predominantly based on healthy volunteers and function effectively as guidelines for the development of assays applied in the diagnosis of disease states. However, they might be of limited use for monitoring disease states or treatments, especially when these factors inherently influence the dynamics of biomarkers and can subsequently alter the biological variation. In case of male castration, testosterone is lowered to < 5% of the reference values and the dynamics, in absence of gonadal production, are predominantly regulated by the adrenal glands. Assessment of biological variation in disease states and during treatments could therefore extend healthy volunteer biological variation data (17, 18).

For these reasons, we estimated the within- and between-subject biological testosterone variation of castrated prostate cancer patients in the Netherlands using a retrospective observational study design and derived APS from these data.

Materials and methods

Specimens

Blood from male prostate cancer patients was drawn by multiple phlebotomists between 7:30 am and 5:00 pm. Left-over patient serum was collected from Rapid Serum Tubes (BD, Franklin Lakes, NJ, United States) in the routine clinical laboratory of the Antoni van Leeuwenhoek Hospital (Amsterdam, The Netherlands), and stored at -20°C until analyzed. Analysis of samples of individual patients were performed in multiple batch runs within two months after collection. Selection of samples was based on testosterone requests for IA analysis by a urologist who was primarily treating localized prostate cancer, an oncologist who was primarily treating metastasized prostate cancer and testosterone requests specifically for LC-MS/MS analysis. Patients were excluded from analysis if the patient 1) objected to inclusion in scientific research, 2) was not diagnosed with prostate cancer, 3) had not been treated with chemical castration only (CAS) or chemical castration in combination with enzalutamide (ENZA) or 4) did not have at least five longitudinal samples. Based on clinical information, samples were categorized as CAS or ENZA. This study was approved by the institutional review board of the Antoni van Leeuwenhoek hospital and the Netherlands Cancer Institute (IRBd18145).

LC-MS/MS assay specifications

Sample preparation was standardized and performed as described previously (19). In brief, 10 µL deuterated internal standard (Testosterone-d5, CDN Isotopes, Pointe-Claire, QC, Canada) was added to calibrator samples and 250 µL QC of patient serum. Liquid-liquid extraction was performed using 1 mL of methyl tert-butyl ether. Dried sample extracts were reconstituted in 100 µL injection working solution (4:6 methanol:water). Before injection, the samples were centrifuged at 18,213 g for 5.0 minutes. Calibrator stock solutions were prepared in methanol. The final calibrator stock solutions were in the range 0.021 – 61.4 nmol/L (eight calibrators). Quality control (QC) levels (0.11, 2.4 and 6.9 nmol/L) were established using serum pools of either human left-over serum or double charcoal stripped fetal bovine serum (FBS). Human left-over serum was collected in the routine clinical laboratory of the Antoni van Leeuwenhoek Hospital (Amsterdam, The Netherlands). FBS (BioConnect, Huissen, The Netherlands) was stripped twice using dextran coated activated charcoal (Merck, Darmstadt, Germany) following a previously published protocol (20).

LC-MS/MS analysis was performed in multiple reaction monitoring mode using a QTRAP6500+ mass spectrometer (MS) (Sciex, Concord, ON, Canada) equipped with an

IonDrive™ Turbo V Source, executed in positive electrospray ionization mode at 600°C. Analytical separation was performed using a Kinetex EVO 1.7 µm C18 column (2.1 mm id, 50 mm) (Phenomenex) and a gradient program of a mobile phase containing water with 0.1% formic acid and 2 mM ammonium acetate, and a mobile phase containing methanol. The gradient starts at 50% methanol and gradually rises to 75% methanol over 2.3 minutes. Subsequently, the methanol percentage increases to 100% in 0.1 minute to wash the column for 0.4 minute. Thereafter, the methanol mobile phase drops to 50% in 0.1 minute to equilibrate the column for the next injection for 0.6 minute. The mobile phase gradient was continuously pumped through the system at a flow rate of 0.6 mL/min. The total run time was 3.5 minutes.

Table 2 gives an overview of the obtained assay imprecision and functional sensitivity characteristics. Total assay imprecision was 7.5 %CV, 5.3 %CV and 5.1 %CV at 0.11, 2.4 and 6.9 nmol/L, respectively. The functional sensitivity of the assay was estimated at 0.025 nmol/L (signal-to-noise ration > 10, imprecision < 20%). The assay was standardized against two serum reference standards, collectively SRM 971 (female and male serum), which were purchased from the National Institute of Standards and Technology (NIST, Gaithersburg, MD, United States).

Biological variation study statistics

Statistical procedures to estimate biological variation parameters were in accordance with previously published guidelines (21, 22). To ensure homogeneity of variance and to identify outliers in duplicate measurements and within-patient samples, Cochran's C test was performed. Identified outlying variances of duplicate samples and within-patients values were excluded from further data analysis (23). To detect between-patient outliers, Reed's Criterion was applied (24). To verify whether patients had steady-state testosterone levels, linear regression was performed for duplicate means at each consecutive time point in days from the first sample collection in the general population for the CAS and ENZA group separately. A group with a slope that included 0 within the 95% confidence interval (CI) was considered to be in steady-state. If a group did not have steady-state of testosterone, all concentrations were transformed applying the inverse of the regression formula. This approach is in accordance with procedures previously reported by the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) working group on biological variation (25). Next, normality of within-patient values and between-patient values was investigated using Shapiro-Wilk and Kolmogorov Smirnov tests (26, 27). Analytical variation (CV_A) was deduced from variances of duplicate measurements; within-patient variation (CV_I) was estimated by an ANOVA of within-patient measurements. As the number of samples per

patient varied, an ANOVA with a nested design was performed to determine the between-patient variation (CV_G). Standard errors of variation parameters were multiplied by 1.96 to estimate the CI.

Differences between mean concentrations and variances in treatment groups were statistically tested with a Students T-test and an F-test, respectively. A p-value below 0.05 was considered significant.

The estimated variation components (CV_A, CV_I and CV_G) were used to calculate the index of individuality (II), RCV and the desirable APS for imprecision (I), bias (B) and total error (TE) (See Table 1). CV_A as determined from variances of duplicate measurements was used to calculate the II, RCV and TE. Estimation of APS was based on a previously published method (28). Data was managed and analysed in R (Version 3.6.3). The R script that was used to generate our results is included in the Supplementary Material (Supplementary Data 3). Graphpad Prism (Version 7) was used for steady-state analysis.

Results

Biological variation and analytical performance specifications

In total, 557 samples collected from 85 male patients between March 2018 and April 2020 met the predefined criteria. Median patient age was 71 years (51 – 86 years). The average number of samples per patient was 6 (5 – 14 samples). Median time between sample collection was 58 days and ranged from 3 to 393 days. For patients, the average study duration was 384 days (80 – 686 days). The Cochran's C test led to the removal of 3 patients that had outlying duplicate samples with heterogeneous variances. In assessing the homogeneity of variance for the CV_I, the Cochran's C test resulted in the exclusion of 10 patients with outlying variances. No patients were identified as outliers by applying the Reed Criterion test. No clear relation was observed between study duration and exclusion as patients that were excluded had a median study duration of 384 days (139 – 639 days). Steady-state analysis detected no significant trends in the CAS and ENZA groups. Concentrations of patients with trends were transformed accordingly. In total, 83 of 557 samples (15 %) and 13 of 85 (15 %) patients were removed from further data analysis. In total, 72 patients (27 CAS patients and 45 ENZA patients) and 474 samples (174 CAS patient samples and 300 ENZA patients samples) were used for estimation of biological variation parameters and APS. Of the remaining patients, 90.3% (92.6% and 88.9% for CAS and ENZA patients, respectively; $p > 0.05$) had normally distributed testosterone levels. Normality could also be assumed for mean patient concentrations ($p > 0.05$). After outlier

removal, testosterone concentrations were significantly higher in the ENZA group (0.318 vs. 0.191 nmol/L, $p < 0.005$). The variances between the groups, as generated by the F-test, were not significantly different ($p = 0.76$).

The biological variation specified for each treatment group is shown in Figure 1; the variation components are shown in Table 3. The mean testosterone concentrations of the ENZA patients had a wider range than those of the CAS patients. Overall, CV_A was estimated at 6.1% (5.9% for CAS patients and 6.3% for ENZA patients). Although CV_I was higher in CAS patients (CAS, 29.6%, 26.5 – 32.7; ENZA, 21.1 %, 19.5 – 22.9) and CV_G was higher in ENZA patients (CAS, 52.0%, 38.2 – 65.9; ENZA, 54.4%, 43.2 – 65.6), their CI overlapped. Both groups showed high individuality (II < 0.6). The RCV was 83.5% for CAS patients and 61.3% for ENZA patients. The desirable assay specifications for I, B and TE are listed in Table 4.

Discussion

In this study, we used a laboratory developed LC-MS/MS-based testosterone assay to monitor testosterone concentrations in (advanced) prostate cancer patients. This method enabled quantitation of suppressed testosterone levels in these patients. We used these results to estimate the biological variation of testosterone in prostate cancer patients treated with chemical castration only or chemical castration in combination with enzalutamide. We found that patients had a broad range of mean testosterone concentrations. This resulted in high CV_G estimates (CAS, 52.0%, 38.2 – 65.9; ENZA, 54.4%, 43.2 – 65.6) In contrast, the CV_I (CAS, 29.6%, 26.5 – 32.7; ENZA, 21.1 %, 19.5 – 22.9) estimates were low compared to the CV_G resulting in a high individuality (II < 0.6). As previously reported, high individuality indicates that the within-subject variation only reflects a small fraction of the population reference interval (29). Therefore, the RCV could be an interesting parameter to apply in investigations into significant longitudinal changes in testosterone during treatment.

Although our study was performed in patients, a superficial comparison can be made with biological variation data on healthy volunteers. Data from studies that were included in the meta calculation by the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) estimate the CV_I at 13.0% (10.9 – 15.1) and the CV_G at 21.3% (17.0 - 83.4) (17, 18, 30-33). The CV_I estimated in chemically castrated prostate cancer patients is significantly higher than the CV_I found in healthy volunteers. Similarly, most studies in healthy volunteers estimated a lower CV_G. However, two previous studies estimated higher CV_G leading to overlapping CI with the CV_G estimated in our study (32, 33). Possible factors contributing to

this difference are the pharmacodynamics of the chemical castration agents and enzalutamide, and the intra-individual regulation of adrenal testosterone. Also, CV_I tends to be higher at low concentration ranges.

In contrast to healthy volunteer biological variation studies, little data has been published for patient groups. Previously, only one study has been performed for testosterone in women with polycystic ovary syndrome (34). The within-patient variation was found to be somewhat higher (24.6% vs. 30.7%, prostate cancer vs. PCOS, respectively) in PCOS patients. Similarly, the between-patient variation appears to be higher (60.3% vs. 64.1%, prostate cancer vs. PCOS, respectively) in PCOS patients. However, in absence of CI around the estimates from the PCOS study no conclusions can be made about the significance of these differences. Yet, these findings do suggest that biological variation is elevated in castrated prostate cancer and PCOS patients compared to healthy volunteers (13.0% and 21.3% median CV_I and CV_G, respectively) (35).

APS derived from the biological variation data are listed in Table 4. Imprecision for all QC pools was in accordance with the desirable imprecision. To our knowledge, no APS for this application have previously been described in literature. In future development of serum testosterone assays specifically designed for castrated prostate cancer patients, these parameters can be used to guide method development and the design of quality control programs.

These data also suggest that steady-state castrate testosterone levels could be used for further diagnostic and clinical studies, as they are relatively stable in relation to the population reference interval. It should be noted that previous studies found indications for sensitive testosterone quantitation as a prognostic biomarker of ADT efficacy. For example, Ryan et al. (36) found that in a large prostate cancer population on abiraterone treatment, higher baseline serum testosterone levels (≥ 0.30 nmol/L) obtained by chemical castration were associated with significantly longer overall survival than lower baseline serum testosterone levels (≤ 0.080 nmol/L). Other reports also suggest a potential role for testosterone as a prognostic biomarker, although they lack sufficient power or appropriate study endpoints (37-39).

Although our findings indicate a potential application of the sensitive LC-MS/MS-based testosterone assay method for castrated prostate cancer patients, certain limitations of our study are apparent. The study did not follow a controlled design, and samples were retrospectively included resulting in varying sampling times, number of samples per patient and times between consecutive samples. This may have resulted in selection bias as, for example, patients were excluded based on number of follow-up samples. Furthermore, using

this study design precludes our results to be regarded as A level according to the Biological Variation Data Critical Appraisal Checklist (BIVAC) previously published by the EFLM (21). However, the relatively large patient population and mean sample size per patient provided adequate study power (40) and should ensure accurate estimation of the within- and between-patient biological variation. It should also be noted that the CV_G estimates were higher than 33%. This indicates that the untransformed data does not follow a normal distribution and is likely to be positively skewed (41). While these estimates can be used as a representation of the dispersion of the data, some caution should be taken in the interpretation of APS calculated with these estimates. Also, we measured the samples in multiple runs, which may have introduced additional between-run variation to the data. However, as the inter-run variation according to the method validation was less than 4%, only a small fraction of variation may have been added. Finally, relatively large numbers of samples and patients were excluded after outlier identification. In addition to the disease state and medication, the blood collection times and the patients' BMI and food intake may have influenced their testosterone concentrations. This may have caused a higher number of outliers to be identified than in a previous study in which outlier analysis was performed before determining biological variation of testosterone in healthy volunteers (30).

In conclusion, we used a laboratory-developed LC-MS/MS-based testosterone assay to estimate the biological variation of testosterone in castrated prostate cancer patients. Furthermore, the observed high individuality indicates that within-patient testosterone levels are stable in relation to the population interval. This suggests that 1) significant individual changes in testosterone as defined by the RCV could be clinically relevant and 2) within-patient testosterone levels could be used to investigate their prognostic or predictive value in prostate cancer hormone therapy. Finally, we provided APS for testosterone quantitation in these patients. These data could now be used as guidelines for developing and quality control design of sensitive LC-MS/MS-based testosterone assays.

Research funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Acknowledgements: We would like to thank Vincent van der Noort for reviewing statistical details and providing essential advice on data analysis.

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Table 1. Parameters and formulae for the variation components and APS estimated in this study.

Parameter	Description	Method/Formula
CV_A	Analytical variation	ANOVA
CV_I	Within-patient variation	ANOVA
CV_G	Between-patient variation	Mixed models ANOVA
II	Index of individuality	$(CV_A^2 + CV_I^2)^{1/2}/CV_G$
RCV	Reference change value	$2.77(CV_A^2 + CV_I^2)^{1/2}$
I	Imprecision specification	$0.5 \times CV_I$
B	Bias specification	$0.25 \times \sqrt{(CV_I^2 + CV_G^2)}$
TE	Total error specification	$(1.65 \times CV_A) + B$

Table 2. **Method imprecision for QC and LLOQ samples.**

CV = coefficient of variation

LLOQ = lower limit of quantitation

QC = quality control

Sample	Concentration (nmol/L)	CV (%)		
		Within-run	Between-run	Total
QC 1	0.11	6.5	3.7	7.5
QC 2	2.4	4.5	2.9	5.3
QC 3	6.9	3.8	3.5	5.1
LLOQ	0.025	12	15	19

Table 3. Ranges, mean values, mean biological variation components (95% CI), II and RCV specified for all patients, and CAS and ENZA groups.

All biological variation components are derived from patient samples.

CAS = Chemical castration only

ENZA = Chemical castration with enzalutamide

CV_A = Analytical variation

CV_I = Within-patient variation

CV_G = Between-patient variation

II = Index of individuality

RCV = Reference change value

Parameter	All (72)	CAS (27)	ENZA (45)
Mean (nmol/L)	0.263	0.191	0.318
Range (nmol/L)	0.047 - 1.44	0.047 - 0.596	0.121 - 1.44
CV_A % (95% CI)	6.1 (5.9 - 6.4)		
CV_I % (95% CI)	24.6 (23.1 - 26.2)	29.6 (26.5 - 32.7)	21.2 (19.5 - 22.9)
CV_G % (95% CI)	60.3 (50.4 - 70.1)	52.0 (38.2 - 65.9)	54.4 (43.2 - 65.6)
II	0.42	0.58	0.41
RCV (%)	70.3	83.5	61.3

Table 4. Desirable assay performance characteristics for testosterone quantitation in castrated prostate cancer patients based on biological variation components.

CAS = Chemical castration only

ENZA = Chemical castration with enzalutamide

I = Imprecision

B = Bias

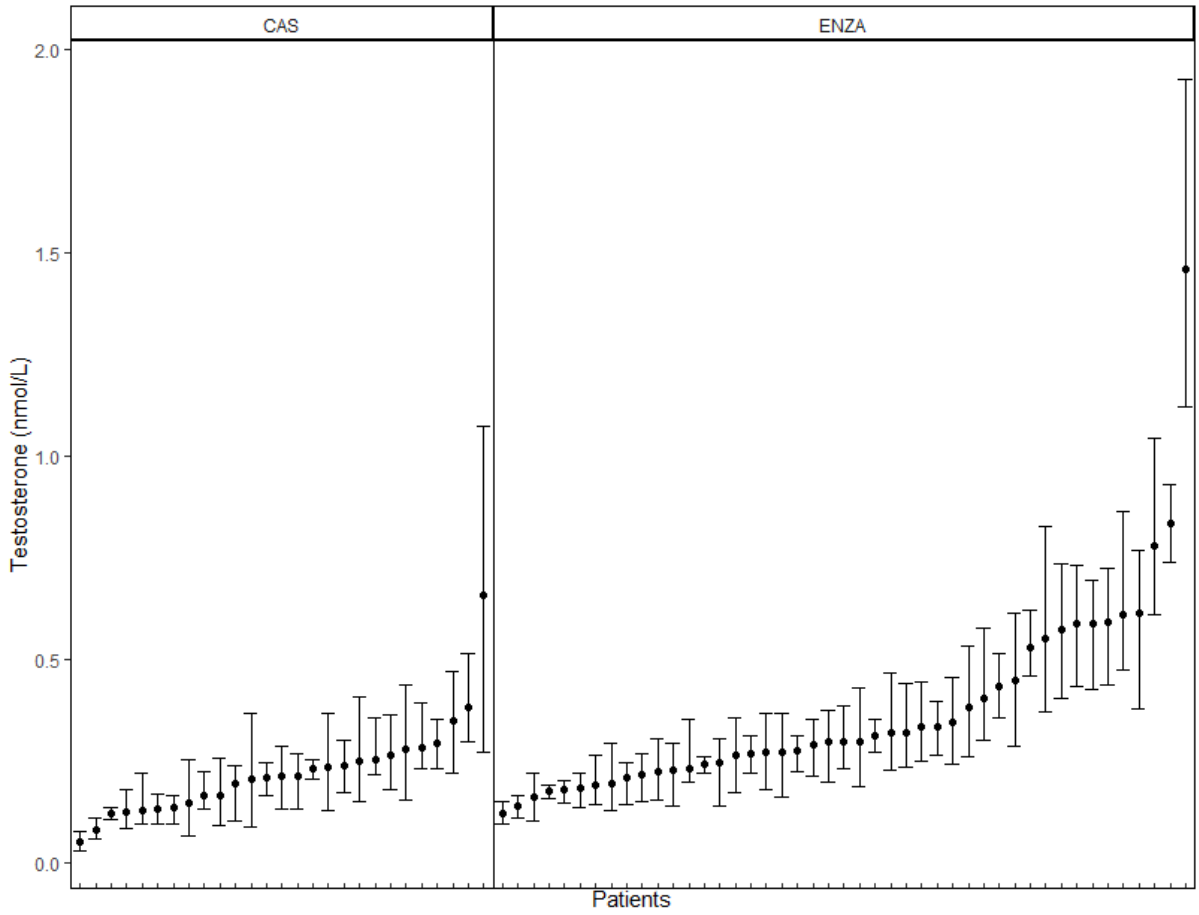
TE = Total error

Group	I	B	TE
All (72)	12.3	16.3	26.4
CAS (27)	14.8	15.0	24.8
ENZA (45)	10.6	14.6	24.9

Figure 1. Mean value and range of testosterone per studied patient.

CAS = Chemical castration only

ENZA = Chemical castration with enzalutamide



Chapter 1.4

An openly available R script for the estimation of biological variation based on EFLM guidelines

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Running title: An R script for biological variation calculation

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Keywords: Biological Variation, R script, EFLM

Abbreviations

APS = analytical performance specifications

BV = biological variation

EFLM = European Federation of Clinical Chemistry and Laboratory Medicine

To the editor:

In laboratory medicine, there is an increasing interest in the biological variation (BV) of measurands. BV is an important parameter to determine analytical performance specifications (APS). Furthermore, it is essential for obtaining reference change values, which are considered useful for the interpretation of longitudinal test results, although relevant limitations have been addressed (1). For these reasons, it is important that BV data is accurate and available. To this end, the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) has recently 1) produced guidelines for performing BV studies, 2) established checklists for the critical appraisal of BV studies, 3) conducted a large series of BV studies and 4) published a website listing meta calculated BV estimates for over 200 markers (2, 3). Although the shift towards standardization of BV studies is essential to increase the accuracy of BV data and greatly appreciated by the authors, the complexity of performing such studies has significantly increased.

In a recent study, our lab estimated the analytical, within- and between-subject BV in castrated prostate cancer patients (4). While previous studies have described statistics to accurately estimate BV (2, 5), we found that translating these papers into a practical methodology using statistical software was associated with many hurdles. For example, the recommended outlier tests are not commonly applied and only available in specific R packages or in specialized and costly software. Furthermore, identification and application of adequate ANOVA models required advanced statistical knowledge. To enable the BV variation in our study, an R protocol was written based on the EFLM recommended BV calculation guidelines. Multiple reviews by a statistician were necessary to verify our statistical approach and coding. To enable other researchers and specialists in laboratory medicine to perform BV studies, we provided our developed R script in the Supplementary Material of this paper (4).

Set-up of R script for BV calculations

A detailed description of the statistical procedures has been reported previously and the script is also openly available at GitHub (https://github.com/LvW-lab/Biological-variation-estimation) (4). In brief, outliers for duplicate measurements and within-subject values are identified and excluded using the Cochrane C test. Outliers in the population range are identified and removed using the Reed's criterion test. Next, steady-state of values is checked using regression analysis and normality is tested within- and between-subject using Shapiro-Wilk tests. Finally, analytical, within- and between-subject BV are estimated with ANOVA. In Table 1, the data structure for statistical analysis is shown. The first column "pat" describes the patients and is numbered in ascending order, whereas the second column

“time.point” indicates the within-subject samples that are analyzed. It is not required to balance the number of samples per subject thus allowing for varying number of within-subject samples. The third column “rep” represents the two replicates that are measured to generate the analytical BV in the data. The last column “meas” lists the values that have been measured by a specific assay. All information in the dataset should be inserted as numeric data and should be saved as a tab separated text file. Notably, this script supports importation of other BV datasets provided that the data has been structured similarly. The script does not handle missing data. The packages GAD (Version 1.1.1), outliers (Version 0.15), tidyverse (Version 1.3.1), VCA (Version 1.4.3), lme4 (Version 1.1-29) and data.table (Version 1.14.2) were used in the script.

While the publication of an open source R script might contribute to an increase in the standardization of BV calculation, we emphasize that further improvements and validation of the script are necessary. At the time of the study the BV results obtained using the script were reviewed by an in-house statistician and briefly compared to the results obtained by the EFLM working group and these were highly comparable for the used dataset. However, no external validation of the script has thus far been performed. Secondly, the script has been written in R and therefore requires at least some basic knowledge of statistical programming, which could limit its use.

Novel guidelines and indexing of critically appraised studies by the EFLM have improved the quality and standardization of BV data greatly. However, methods to perform the statistical analysis of BV study data have not become publically available and it can be challenging for labs to perform these calculations. This letter addresses this issue and provides an openly available R script for the estimation of BV. Finally, we emphasize that further standardization and availability of these statistical procedures, preferably supported by the EFLM, would further enable accurate BV estimations.

Conflict of interest

HVR has 1) received consulting fees and honoraria for Huvaros B.V., has 2) issued two patents (PCT/NL2016/050315 and NL2019/079641), has 3) stock options in Huvaros B.V. and SelfSafeSure Blood Collections B.V. and has 4) reveived material from Roche Diagnostics for the DEDICATION-1 clinical study.

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Table 1. A fictional dataset containing two patients. The table has been generated using Excel and the variables consist of numeric values.

pat	time.point	rep	meas
1	1	1	2.311
1	1	2	2.315
1	2	1	2.328
1	2	2	2.257
1	3	1	1.888
1	3	2	2.008
1	4	1	2.71
1	4	2	2.646
1	5	1	2.3
1	5	2	2.32
2	1	1	3.861
2	1	2	4.142
2	2	1	4.617
2	2	2	4.711
2	3	1	3.063
2	3	2	2.747

Chapter 1.5

Simultaneous analysis of E1 and E2 by LC-MS/MS in healthy volunteers: estimation of reference intervals and comparison with a conventional E2 immunoassay

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Running title: LC-MS/MS-based estrogen analysis in healthy volunteers

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Abbreviations:

17-OHP	17-hydroxyprogesterone
CV	Coefficient of variation
DHEA	Dehydroepiandrosterone
E1	Estrone
E2	Estradiol
IA	Immunoassay
IS	Internal standard
LLOQ	Lower limit of quantitation
LOD	Limit of detection
LC-MS/MS	Liquid chromatography tandem-mass spectrometry
MRM	Multiple reaction monitoring
NH4F	Ammonium fluoride
QC	Quality control
UHPLC	Ultra high-performance liquid chromatographer

Abstract

Monitoring estrogen levels, especially estradiol (E2), is amongst others important for determining menopausal status and guidance of breast cancer treatment. We validated a serum E2 and estrone (E1) liquid chromatography tandem-mass spectrometry assay (LC-MS/MS) suitable for quantitation in human subjects. In addition, we compared our method with an E2 immunoassay (IA) and established preliminary reference values. Validation parameters were within the predetermined acceptance criteria. Assay linearity ranges were 4-1500 pmol/L for E1 and 4-2500 pmol/L for E2. Imprecision ranged from 7.4 to 9.6%. The lower limit of quantitation for E2 (8.0 pmol/L) was 11.4 times lower than the IA. The method comparison revealed differences in E2 quantitation up to 155% between both methods. The method allowed quantitation of E1 in all healthy volunteers, while E2 could not be detected in 95% versus 40% of the post-menopausal women using IA and LC-MS/MS respectively. Male, pre-, peri- and post-menopausal female reference values were estimated. An LC-MS/MS based method combining E1 and E2 analysis was validated with superior E2 analytical sensitivity when compared to the IA.

Key words: LC-MS/MS, immunoassay, estrogen, estrone, estradiol, menopause, reference values

Introduction

In women, estrogens are important for the development and upkeep of the reproductive system, secondary sex characteristics, menstrual cycle and pregnancy. The two most prevalent estrogens are estrone (E1) and estradiol (E2), with E2 being the most biologically active (1). Laboratories quantitate circulating E2 levels to aid in the diagnosis of amongst others; female fertility disorders, ovarian hyper stimulation in the context of in-vitro fertilization, determination of menopausal status and gynecomastia in males (2). For breast cancer patients, assessment of ovary function and menopausal status is essential to guide systematic hormonal treatment. In this context, E2 quantitation is used to confirm proper suppression of ovary function in pre- and peri-menopausal patients to assure treatment efficacy of aromatase inhibitors (3, 4). E1 is not commonly measured in laboratories, despite being the most abundant circulating estrogen in post-menopausal women (1, 5, 6).

Physiological levels of estrogens, especially in postmenopausal women, are low (E1, < 148 pmol/L; E2, < 77 pmol/L) and highly sensitive assays are required to allow quantitation (5, 6). For E2 analysis, laboratories mostly rely on cost effective and high throughput immunoassays (IA) offering considerable sensitivity. However, in most postmenopausal women, specifically for breast cancer patients receiving aromatase inhibitors, circulating E2 levels measured with an IA are non-quantitable. Furthermore, IA are known to lack specificity in low concentrations due to cross-reactivity potentially resulting in unreliable quantitation of E2 (7-10). Liquid chromatography tandem-mass spectrometry (LC-MS/MS) based methods are considered best practice for steroid analysis and can quantitate estrogens in low pico-molar concentrations with increased specificity compared to immunoassays (11). Although LC-MS/MS estradiol methods offer obvious advantages, they are labor intensive and require additional expertise to perform correct analysis (12, 13).

In this study, we present the validation of an LC-MS/MS based method for the simultaneous quantitation of E1 and E2. Furthermore, reference values were estimated and E2 results were compared with the in-house E2 IA.

Materials and methods

Reagents, standards and specimens

Standards including 17-hydroxyprogesterone (17-OHP), activated charcoal, androstenedione, cortisol, dehydroepiandrosterone (DHEA), epitestosterone, E1, E2, progesterone and testosterone were purchased from Sigma Aldrich (St. Louis, MO, USA).

Deuterium-labeled internal standards (IS) estrone-2,4,16,16-d4 and 17β-estradiol-2,4,16,16-d4 were obtained from CDN Isotopes (Pointe-Claire, Quebec, Canada). All materials were of the highest analytical grade. Ammonium fluoride (NH4F) was purchased from Merck Chemicals (Burlington, MA, USA). All standards and internal standards were dissolved in DMSO to a concentration of 10 mmol/L. Calibrator stock solutions were established in methanol and added to saline solution in the following ranges using seven calibrators for each analyte: 4 – 1500 pmol/L, E1 (4, 10, 25, 100, 250, 750, 1500 pmol/L); 4 – 2500 pmol/L, E2 (4, 10, 25, 100, 250, 1000, 2500 pmol/L). IS working solution was prepared in methanol at a final concentration of 25000 pmol/L for both estrogens. Low, medium and high serum reference materials were purchased from Sigma Aldrich (BCR certified, St. Louis, MO, USA). Healthy volunteer serum was collected from patient spouses between 2004 and 2017 during hospital visits. The study was approved by the institutional review board of our hospital and a signed informed consent from healthy volunteers was acquired before blood withdrawal.

Sample preparation

Blood samples were collected by venipuncture procedure in a rapid serum tube (Becton Dickinson (BD), Franklin Lakes, NJ, United States) and serum was used as sample matrix. Aliquots of 250 µL QC or patient serum were added to 3 mL (i.d. 10.5 mm) glass test tubes. To each sample, 10 µL IS working solution was pipetted to a final concentration of 961 pmol/L. Similar to a previously described method, estrogens were extracted by mixing samples with 1 mL Hexane:Ethyl Acetate 9:1 (v:v) for 30-min (14). Thereafter, the organic phase was separated and collected in glass injection vials by snap freezing the aqueous layer. Subsequently, the organic phase was dried using a SpeedVac concentrator. Dried extracts were reconstituted in 75 µL freshly prepared injection working solution (methanol:water 1:4, v:v). Before injection, samples were briefly shaken and spun down.

LC-MS/MS

Analysis was executed in multiple reaction monitoring (MRM) mode using a QTRAP6500+ mass spectrometer (Sciex, Concord, ON, Canada). Ionization of estrogens was achieved with an IonDrive™ Turbo V Source applied in negative electrospray ionization mode at 650 °C. Two mass transitions were monitored for E1 (m/z 269 → 145; m/z 269 → 143) and E2 (m/z 271 → 145; m/z 271 → 143) and one for each IS (d4-E1, m/z 273 → 147; d4-E2, m/z 275 → 147). The Shimadzu Nexera X2 ultra high-performance liquid chromatographer (UHPLC)(Columbia, MD, USA) was employed to provide a flow of 0.6 mL/min through a Kinetex 1.7 µm phenyl-hexyl column (2.1 mm id, 50 mm, Phenomenex). Column temperature was maintained at 30 °C. To chromatographically separate the estrogens a gradient mobile phase was established composed of 50 µM NH4F in water (phase A) and of

50 µM NH4F in 5% water and 95% MeOH (phase B). NH4F was added to the mobile phase to increase ionization efficiency (15, 16). A linear gradient of 2 min from 40% phase B to 100% phase B was used to separate analytes. Afterwards, the column was flushed with 100% phase B and equilibrated for 1 min at the starting conditions. A flow of 0.6 ml/min was sustained over a total run time of 3.5 min. Sample injection volume was set at 50 µL. The concentration was calculated based on the peak area ratio of the analyte to the internal standard in relation to the calibration curve equation. For all patient samples, E1 and E2 analysis was performed in duplicate and final results were obtained by mean concentration calculations.

E2 IA

For E2, healthy volunteer samples were measured using a third generation E2 immunoassay on a Cobas E601/602 system (Roche diagnostics, Basel, Switzerland). The calibration range was 18.4-11010 pmol/L with a LLOQ of 91.8 pmol/L and limit of detection (LOD) of 18.4 pmol/L. Calibrators, internal- and external quality control samples were measured routinely as specified by the manufacturer instructions for use.

LC-MS/MS assay validation

(Pre-) analytical method validation was performed and included imprecision, lower limit of quantitation, trueness, sample stability, linearity, matrix effect and extraction recovery, carry-over and interference. Imprecision was determined by analysis of three serum pools in quadruplicate for ten consecutive runs on separate days containing concentrations distributed over the measuring range. The lower limit of quantitation was determined by measuring three serum pools of spiked double charcoal stripped fetal bovine serum (Approximately 6, 8 and 10 pmol/L for both E1 and E2) in duplicate for six consecutive runs on separate days with analyte peaks showing a S/N > 10. The mean of the pool containing the lowest concentration and meeting the predefined criteria was accepted as LLOQ. Criteria for imprecision and the lower limit of quantitation were a total coefficient of variation (CV) below 10% and 20%, respectively. For E2, assay trueness was determined by measuring medium and high serum reference material in triplicate and low serum reference material in duplicate for four consecutive runs on separate days. A bias below 5% was considered acceptable. For E1, calibration was performed in triplicate with a European reference standard. Sample stability was evaluated for three serum pools at -20 °C (2 months), 4 °C (2 weeks) and 20 °C (1 week). Linearity was evaluated at 7 levels across the measuring range. Polynomial regression was performed and linearity fit was tested in EP Evaluator (Version 12.2). Matrix effects and extraction recovery were determined by pre- and post-extraction

standard addition. Carry-over effects were investigated in three blank samples after injection of three consecutive high calibrator samples (E1, 1500 pmol/L; E2, 2500 pmol/L). Interference was tested by analyzing three serum pools spiked with 17-hydroxyprogesterone (5 nmol/L), 17α-estradiol (0.5 nmol/L), 17α-ethynylestradiol (0.5 nmol/L), anastrozole (250 nmol/L), androstenedione (5 nmol/L), cortisol (500 nmol/L), dehydroepiandrosterone (50 nmol/L), dihydrotestosterone (5 nmol/L), epitestosterone (50 nmol/L), exemestane (100 nmol/L), letrozole (100 nmol/L), prednisolone (1 nmol/L), prednisone (1000 nmol/L), progesterone (5 nmol/L), tamoxifen (1000 nmol/L), testosterone (50 nmol/L), hemoglobin (1 mmol/L), bilirubin (50 µg/mL) and intralipid (1%). A recovery within ± 10% was considered acceptable for evaluating sample stability and interference. Validation was in accordance with previously published guidelines (17).

Estimation of reference values

To estimate the reference values, serum samples from healthy males (n = 124), healthy females aged 18-40 years (n = 121), healthy females aged 41-60 years (n = 128) and healthy females aged ≥ 61 years (n = 122) were separately studied. Sample size was based on recommendations made by the Clinical and Laboratory Standards Institute (CLSI EP28A3)(18). The age groups in healthy females were chosen to represent premenopausal, perimenopausal and postmenopausal females. During hospital visits of cancer patients, their accompanying spouses were asked to give blood samples. The inclusion criterion was that the volunteer had never been diagnosed with cancer and no further information regarding menopausal status and/or the use of contraceptive and hormonal drugs was obtained. Blood was collected between 7:00 am and 5:00 pm. For estimation of the reference values, normality of distributions was tested by generating q-q plots. Accordingly, skewed distributions were log-transformed. Subsequently, 2.5^{th} and 97.5^{th} percentiles were calculated and applied as lower and upper limits in our analysis, respectively. Mann-Whitney U tests were applied to demonstrate significance. Statistics were performed either with GraphPad Prism (Version 7.03) or RStudio (Version 1.3.1093).

E2 method comparison

Method comparisons were performed for male and female samples separately and analyzed by Passing-Bablok regression and relative difference plots generated in Analyse-it (Version 5.10.9). Furthermore, insights in the number of non-quantitable patient samples for both methods (<LLOQ) were studied for the next subgroups; men, women aged < 41 years, women aged > 40 and < 61 years and women aged > 60 years. A McNemar test was

performed on paired binominal data in RStudio (Version 1.3.1093) to test significant differences in quantitable samples. A $p < .05$ was considered statistically significant.

Results

LC-MS/MS assay validation

A typical chromatogram is illustrated in Figure 1. Retention times were 1.71 and 1.61 minut for E1 and E2, respectively. No co-eluting peaks were observed during validation experiments. An overview of the assay imprecision and LLOQ characteristics are displayed in Table 1. Assay imprecision ranged from 7.4 - 9.6 % for both estrogens in all QC pools. The LLOQ was determined at 6.9 pmol/L for E1 and 8.0 pmol/L for E2. E1 and E2 were stable (90 – 110% recovery) in all tested storage conditions (seven days at 20 °C, one and two weeks in an RST tube at 4 °C for E2 and E1 respectively, and two weeks at – 20 °C). Furthermore, prolonged storage (> 1 year) of QC samples at – 20 °C did not significantly affect recovery. The assay linearity showed a correlation (Spearman's correlation coefficient, $r^2 \geq 0.995$) for both estrogens and demonstrated the best fit for first order polynomial regression. Extraction recoveries were above 95% for E1 and E2, and the matrix effect was 84% and 69% for E1 and E2 respectively. No carry-over signal was detected in the blank samples that were measured after the three highest calibrator samples. No significant interference from 17-hydroxyprogesterone, 17α-ethynylestradiol, anastrozole, androstenedione, cortisol, dehydroepiandrosterone, dihydrotestosterone, epitestosterone, exemestane, letrozole prednisolone, prednisone, progesterone, tamoxifen, testosterone was detected. Furthermore, addition of 1 mmol/L hemoglobin, 50 µg/mL bilirubin and 1% intralipid did not affect estrogen quantitation. However, addition of 0.5 nmol/L of 17α-estradiol resulted in a quantitation recovery ranging from 133 to 2419 % in three different serum pools for E2. No interference by 17α-estradiol was observed for E1. Further details on sample stability and interference are listed in the Supplemental Material (Table 1 and 2).

Estimated reference values

The obtained estrogen concentrations in the healthy volunteers are presented in Figure 2 and the estimated reference values are presented in Table 2. For all E2 groups, lower interval limits were calculated below the LLOQ. E1 was quantitable for almost all 495 analyzed samples, whereas E2 concentrations were occasionally (67/495, 14%) below the LLOQ, especially for females aged ≥ 41 years (52/250, 21%). For E1 and E2, median levels were significantly lower in females aged above 60 years compared to females aged below 41 years.

E2 method comparison

For 214 healthy volunteer samples a separate comparison was performed for males and females. The obtained results together with the Passing-Bablok regression are presented in Figure 3A and 3B. For male samples, the slope did not deviate significantly from 1 (0.93 to 1.7 95%CI), whereas the intercept showed a significant difference from 0 (14.5 to 46.6 95%CI). In addition, slope and intercept for female samples demonstrated both significant differences from 1 (1.15 to 1.30 95%CI) and 0 (21.6 to 30.6 95%CI), respectively. Difference plots are presented in Figure 3C and 3D. Differences up to 155% (Male samples) and 138% (Female samples) were observed.

In table 3, the number of non-quantitable E2 levels are listed for the Roche IA (LLOQ, 91.8 pmol/L) and the LC-MS/MS method. For all samples and male samples, the LC-MS/MS had significantly lower non-quantitable samples ($p < .001$). Furthermore, in all individual female groups and in the male group, we found that the number of non-quantitable samples was significantly lower. Notably, we observed E1 was quantitable in all groups (See Figure 2).

Discussion

Here, we successfully validated an LC-MS/MS assay for measurement of E1 and E2 allowing over 11 times more sensitive E2 quantitation than the in-house routinely applied IA. Furthermore, E1 concentrations were quantitable in all male and female samples. To investigate whether our method can quantitate estrogens in healthy volunteers, we determined preliminary reference values for males aged at least 18 years, females aged 18-40 years, females aged between 41 and 60 years and females aged at least 61 years. To this end, in-house biobank samples were used in the absence of information on the female subjects' menopausal status, menstrual cycle period or use of birth control pills. Therefore, no definite reference values for both estrogens in females in regard to menstrual cycle period and menopausal status could be determined. We separated female samples by age to assess the effect of the menopause on the circulating concentrations of E1 and E2. Although onset of menopause is known to be influenced by race, ethnicity and lifestyle factors, the overall median age at menopause is between 50 and 52 years with the vast majority of women being premenopausal before the age of 45 and most being postmenopausal after the age of 55 years (19-21). To increase the chance that the large majority of the pre- and post-menopausal females were indeed in this menopausal stage, broad age cut-offs at 40 and 60 years for peri-menopausal female subjects were selected. Significant differences in estrogen levels between premenopausal and postmenopausal as defined by our age classification were observed for both E1 and E2.

In literature, well-established estrogen reference values using LC-MS/MS methodology are relatively scarce. Four studies have previously described reference values for E1 and E2 (5, 6, 10, 22), while another study recently published reference intervals only for E2 (23). For both estrogens, considerable variations in reference values are observed. This could be explained by 1) differences in population selection and characterization, 2) poor standardization between methods, 3) selection of direct or derivatization procedures and 4) various statistical approaches in determination of the reference range (i.e. 95%CI, IQR or whole range) (24, 25).

Additionally, we investigated the differences in E2 quantitation by our in-house routine IA and the newly developed LC-MS/MS method in healthy volunteers. The first observation was that the LC-MS/MS was able to quantitate E2 levels in a significantly larger number of samples in all groups (p-values below .001). In the second analysis, relative differences up to 155% were detected, especially in the lower concentration ranges found in males and females aged above 60 years old. As our E2 LC-MS/MS method has superior specificity over the IA and was standardized against certified reference material, these findings suggest unreliable quantitation of E2 by the IA in lower concentration ranges. This could potentially be an issue for breast cancer patients in which ovarian function assessment is necessary to determine whether aromatase inhibitor treatment is appropriate (3, 4).

Notably, E1 levels were quantitable in all the studied females, whereas E2 levels could not be quantitated in 66 of the 371 female samples (18%), occasionally samples from females aged above 60 years (32/122, 26%). This can be explained by a relatively high production rate of E1 in peripheral tissues through aromatization of androstenedione and desulfatization of estrone sulfate in postmenopausal women of which the ovarian production of estrogens, predominantly E2, has largely stopped (26). In breast cancer treatment, aromatase inhibitors are primarily prescribed for postmenopausal women and target this residual estrogen production (27, 28).

Interestingly, an early study showed that E1 levels quantitated using a radioimmunoassay in peripheral tissues are correlated with circulating E1 levels (29). Although another early study found no correlation using a radioimmunoassay (30), two recently published articles confirmed the former results applying LC-MS/MS technology (26, 31). Possibly, for the above-mentioned reasons, left-over estrogen, or more specifically circulating E1 concentrations, can be used as a prognostic or even a predictive biomarker for breast cancer patients.

Although this study exhibits significant discrepancies between the Cobas E2 IA and our LC-MS/MS E2 method in healthy volunteers, two limitations should be noted. The limited information on the healthy volunteers for the estimation of reference values may have affected interpretation of our data. Further information such as body mass index, intake of medication, menopausal status and menstrual cycle period could explain data outliers. Another limitation is the lack of sensitivity of our method. Recently published methods have demonstrated lower LLOQs without using chemical derivatization (10, 23). However, these methodologies require solid-phase extraction and 2D LC which substantially increases run time and analytical complexity. We designed the method to be as simple as possible to enable high-throughput application.

In summary, we have successfully validated a serum estrogen LC-MS/MS method that was considered suitable for application in human subjects. Significant discrepancies were demonstrated in low circulating E2 levels with the in-house IA. Furthermore, using biobank samples, we estimated of the reference values for pre-, peri- and post-menopausal women and in males. While these results clearly show the technical benefit of using LC-MS/MS-based estrogen analysis instead IA technology, future studies are necessary to determine its potential in breast cancer patients.

Acknowledgements

None.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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Table 1. Method imprecision and LLOQ

LLOQ = lower limit of quantitation

CV = coefficient of variation

		E1	E2
Low			
	Mean (pmol/L)	61.0	54.2
	Within run CV (%)	7.5	9.2
	Between run CV (%)	3.0	2.8
	Total CV (%)	8.1	9.6
Medium			
	Mean (pmol/L)	401	477
	Within run CV (%)	7.6	6.6
	Between run CV (%)	2.3	3.3
	Total CV (%)	7.9	7.3
High			
	Mean (pmol/L)	1034	1204
	Within run CV (%)	6.9	7.1
	Between run CV (%)	2.9	2.5
	Total CV (%)	7.5	7.6
LLOQ			
	Mean (pmol/L)	6.9	8.0
	Total CV (%)	9.0	8.6

Table 2. Estimated reference intervals in healthy male and female volunteers based on age groups.

E1 = estrone

E2 = estradiol

	Estimated reference values (pmol/L)	
Population	E1	E2
Males ≥ 18 years (n=124)	40 - 143	9 - 114
Females 18 - 40 years (n=121)	25 - 543	≤ 1146
Females 41 - 60 years (n=128)	24 - 519	≤ 1164
Females > 60 years (n=122)	21 - 151	≤ 47

Table 3. Number of non-quantitable E2 samples using IA and LC-MS/MS assay.

The % represent percentage of undetectable number of samples within a group (Total, male, females aged below 41 years, females aged between 40 and 61 years and females aged above 60 years). Differences were statistically tested (McNemar, $p < .05$ was significant).

E2 = estradiol

IA = immunoassay

LC-MS/MS = liquid chromatography tandem-mass spectrometry

LLOQ = lower limit of quantitation

	LC-MS/MS	IA (LLOQ = 91.8 pmol/L)	
	No. (%)	**No. (%)**	**p-value**
All samples (n = 214)	41 (19)	126 (59)	< .001
Male (n = 63)	0 (0)	30 (48)	< .001
Female < 41 years (n = 44)	7 (16)	18 (41)	< .001
Female < 61, > 40 years (n = 67)	16 (24)	40 (60)	< .001
Female > 60 years (n = 40)	16 (40)	38 (95)	< .001

Figure 1. Chromatogram of a healthy volunteer sample containing 307 and 158 pmol/L of E1 and E2, respectively. Retention times were determined at 1.71 min for E1 and 1.61 min for E2. Total run time is 3.5 min.

E1 = estrone

E2 = estradiol

d4-E1 = estrone-2,4,16,16-d4

d4-E2 = 17β-estradiol-2,4,16,16-d4

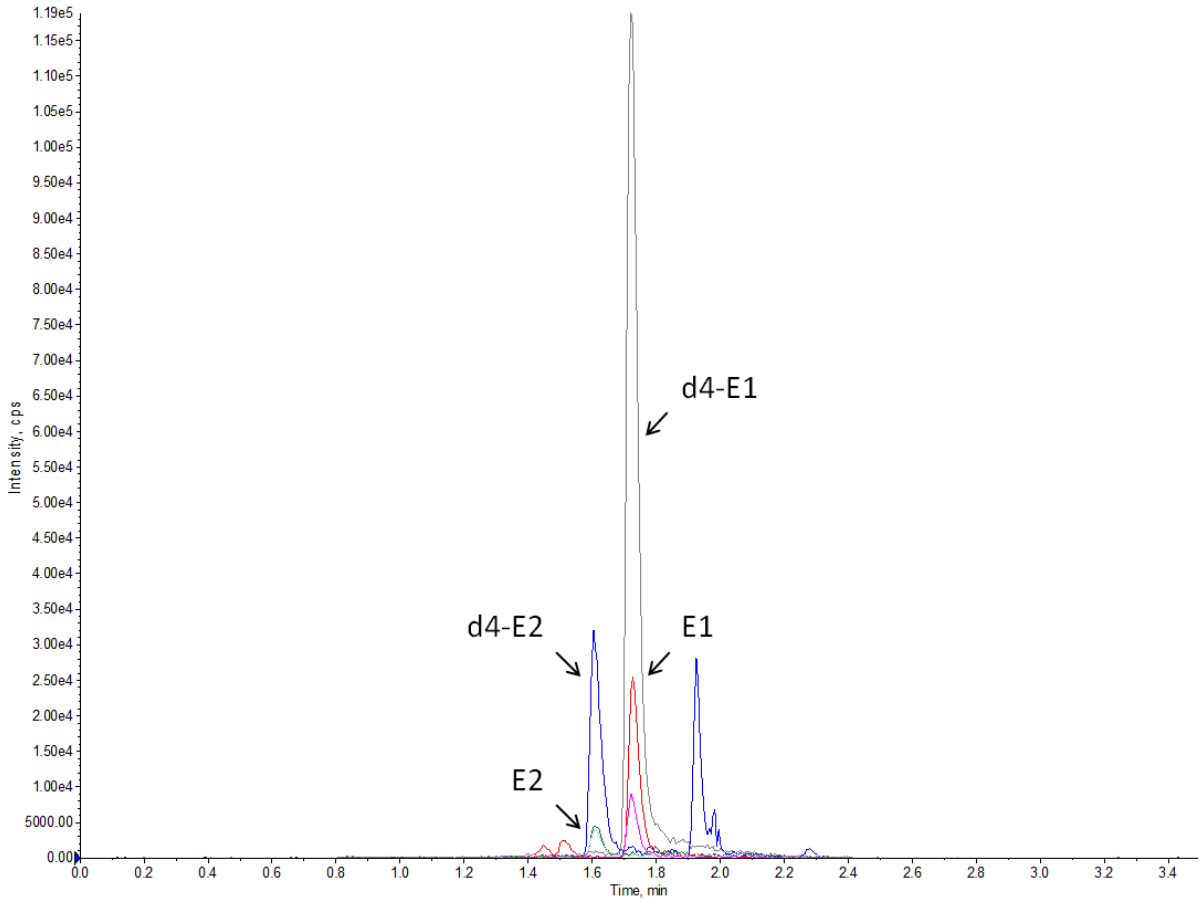


Figure 2. Scatterplots of E1 and E2 for all healthy volunteer groups.

Medians are highlighted with grey lines. Estrogen concentrations were plotted on a logarithmic scale to enable visual comparison between groups; **** p < .0001.

E1 = estrone

E2 = estradiol

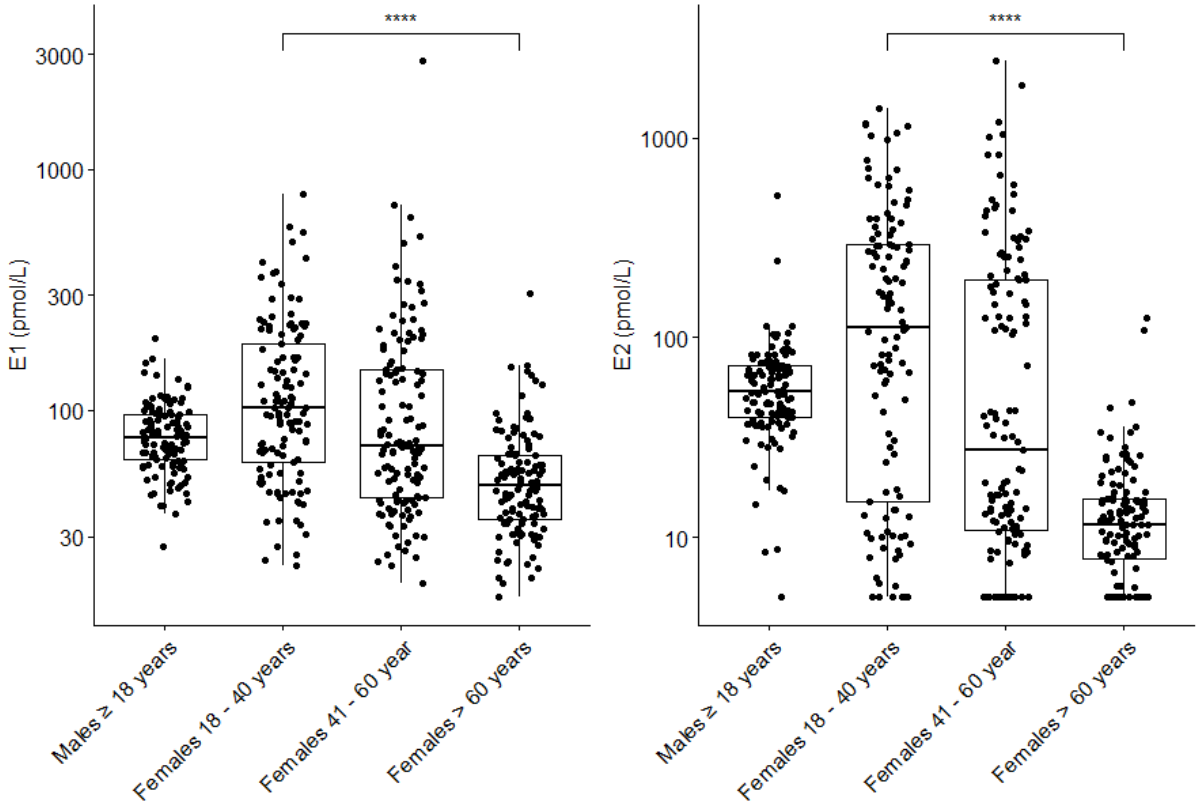
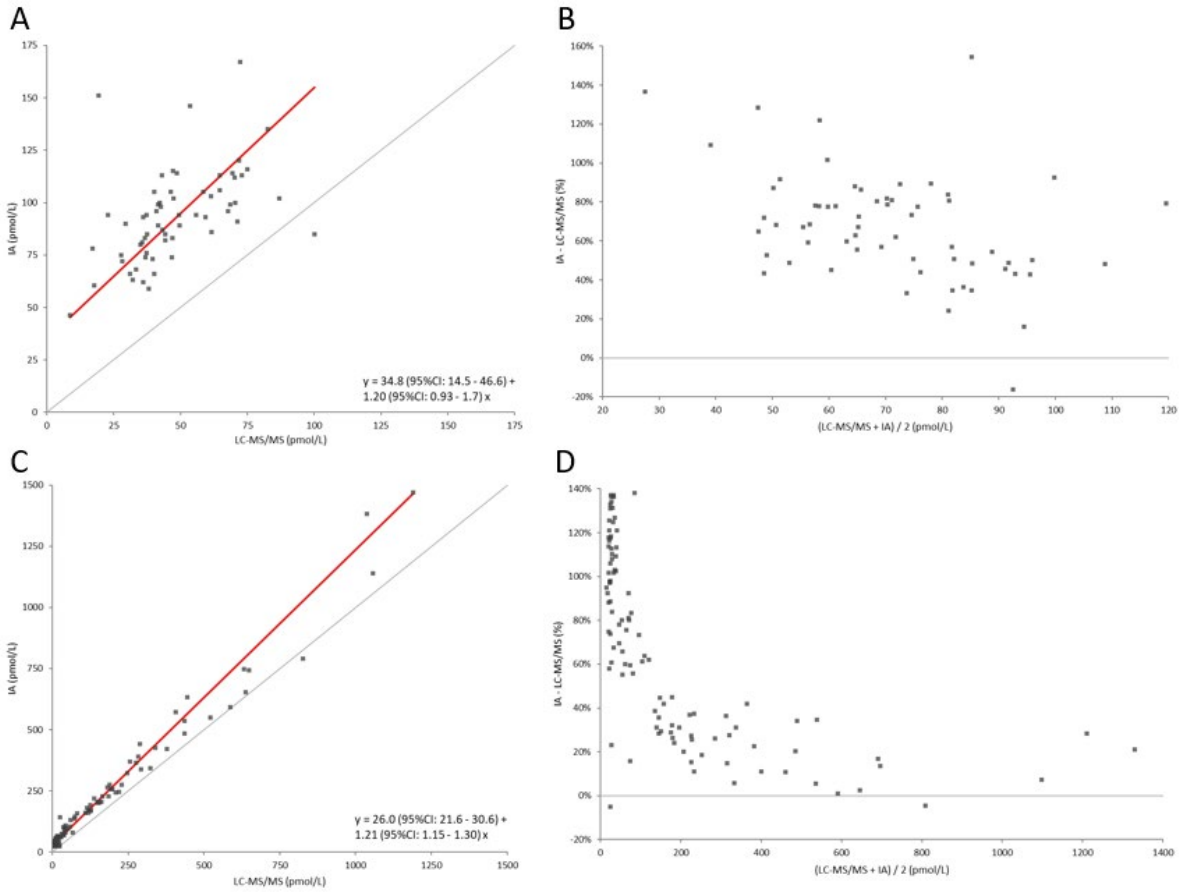


Figure 3. Passing-Bablok regressions and relative difference plots of the method comparison between the LC-MS/MS assay and the 3rd generation E2 assay on a Cobas E601/602 system. A and show all healthy male samples, whereas C and D shows all healthy female samples.

IA = immunoassay

LC-MS/MS = liquid chromatography tandem-mass spectrometry

E2 = estradiol



Section 2: Clinical validation of testosterone analysis by LC-MS/MS in prostate cancer patients

Chapter 2.1

Testosterone analysis in prostate cancer patients

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Running title: testosterone analysis prostate cancer patients

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Abbreviations:

1D	One-dimensional
2D	Two-dimensional
ADT	Androgen deprivation therapy
AR	Androgen receptor
BAT	Bipolar androgen therapy
cAMP	Cyclic adenosine 3',5'-cyclic monophosphate
CAS	Chemical castration only
CI	Confidence interval
CLIA	Chemiluminescent immunoassay
CRPC	Castration resistant prostate cancer
CYP17A1	Cytochrome P450 17A1
DES	Diethylstilbestrol
EFLM	European Federation of Clinical and Laboratory Medicine
ENZA	Chemical castration with enzalutamide
FS	Functional sensitivity
GC	Gas chromatography
HSPC	Hormone sensitive prostate cancer
IA	Immunoassay
IQR	Interquartile range
IVD	In vitro diagnostics
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography tandem –mass spectrometry
LH	Luteinizing hormone
LHRH	Luteinizing hormone-releasing hormone
LLOQ	Lower limit of quantitation
LOD	Limit of detection
MS	Mass spectrometry
NOADT	Without ADT
RIA	Radioimmunoassay
SD	Standard deviation
VACURG	Veterans Administration Cooperative Urological Research Group

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Abstract

Testosterone is an essential steroid hormone associated with a wide variety of biological processes in humans, and especially in males. In prostate cancer, androgen signaling is an important driver of tumor cell growth. Depletion of gonadal testosterone, achieved by surgical or chemical castration, prevents androgenic signaling and temporally reduces, stops or reverses tumor growth before inevitable progression to castration resistant prostate cancer occurs. Additional treatment strategies targeting androgenic signaling have become available, although these are without curative intent. While circulating testosterone levels are also associated with disease risk and potential clinical utility, their main use in the clinical lab is monitoring adequate castration and subsequent resistance to therapy. Adequate castrate levels of testosterone are currently based on over 50 year old double-isotope derivative assays and are disputed in light of automated immunoassay (IA) analysis. The debate has been further fueled with the introduction of mass spectrometry-based assays for testosterone, offering a substantial increase in sensitivity and specificity in contrast to IA. In this review, we will first discuss testosterone regulation and androgen deprivation therapy in relation to prostate cancer. Next, we will provide an overview of the developments in testosterone analysis for monitoring adequate castration and resistance to therapy by various assay types. This information will be put into context of current clinical practice and potential future clinical utilities. Finally, clinical and research recommendations based on these findings are presented.

1. Introduction

Prostate cancer is the most prevalent cancer in males and contributes substantially to mortality (1). While localized prostate cancer has a good prognosis with 5-year overall survival approaching 100%, advanced prostate cancer is associated with an unfavorable 5-year overall survival estimated at 40%. Advanced prostate cancer is predominantly treated with therapeutics targeting the androgen signaling pathway, a target famously discovered in 1941 by Charles Huggins and Clarence Hodges (2). They found that testosterone and its metabolites are essential for prostate cancer proliferation, and that surgical castration or injection of estrogens decreased or even reversed tumor growth. Since then, various treatment strategies have been developed aiming at lowering testosterone. One of the most important developments has been the introduction of chemical castration by luteinizing hormone-releasing hormone (LHRH) agonists. These chemicals hamper the gonadotropic signaling responsible for the induction of testosterone production in the gonads effectively and improved survival drastically, although progression to castration resistant prostate cancer (CRPC) is unavoidable.

To assess whether progression can be ascribed to resistance to therapy, and not inadequate castration, serum testosterone levels are monitored. Studies in the 1960s and 1970s using double-isotope derivative assays reported that testosterone levels below 1.7 nmol/L indicate adequate castration (3, 4). However, more recent studies using advanced automated immunoassays (IA) indicate that a lower cut-off is probably more appropriate (5, 6). In medical laboratories, different assay techniques are used to quantitate castrate levels of testosterone in prostate cancer patients. To enable short assay times and low costs, automated random access automated IA are widely applied for testosterone analysis in routine clinical practice (7). Notably, IA lack sensitivity and suffer from cross-reactivity in the lower concentration ranges potentially generating unreliable results (8-10). As an alternative, liquid chromatography tandem-mass spectrometry (LC-MS/MS)-based testosterone analysis has been increasingly introduced incorporating enhanced detection and separation techniques (11-13).

Previous studies comparing testosterone IA and LC-MS/MS assays in women (14, 15) and neonates (16) have been performed demonstrating strong and clinically relevant discrepancies between results. No method comparison data has been published for castrated prostate cancer patients yet (17). However, it is expected to reveal similar results. In the context of these discrepancies, LC-MS/MS is considered the gold standard in female and pediatric steroid analysis by prominent journals publishing studies in the field of endocrinology (12, 18).

Although mass spectrometry-based analysis has clear advantages over IA, some limitations are apparent. Development and implementation of these assays require additional expertise and are often costly. Furthermore, methods are associated with relatively long sample pretreatment procedures and assay times. This has sparked the debate whether LC-MS/MS should be applied more widely for castrated prostate cancer patients. The goal of this review is to provide an overview of testosterone analysis in the context of prostate cancer and discuss the various arguments used in selecting a proper assay technique.

2. Gonadal testosterone regulation

Although in males low amounts are produced by the adrenal gland through adrenocorticotropic hormone (ACTH) stimulation (19), testosterone is primarily derived from Leydig cells that are located within the gonads. Leydig cells are responsible for approximately 95% of the total body testosterone production and were first characterized by Franz von Leydig in 1850 (20). At this time, not much was known on the biology of the testis and especially their crucial role in steroidogenesis was not yet elucidated. More than a century later, in the 1950s and 1960s, studies were conducted showing that interstitial cells of the testis convert cholesterol into progestagens and subsequently metabolize these progestagens into androgens. Around the same time, Hall and Eik-Nes discovered that rabbit testis produced testosterone under influence of luteinizing hormone (LH), which is regulated by the hypothalamic-pituitary-gonadal axis (21).

In this regulatory system, LHRH is encoded by the *GNRH1 gene and* secreted by the hypothalamus (22). Subsequently, LHRH binds G-protein coupled receptors activating a second messenger signalling pathway responsible for LH production (23). Hereafter, LH is secreted by the pituitary and binds the LH receptor present on the membrane of Leydig cells. After activation of the LH receptor, the cyclic adenosine 3',5'-cyclic monophosphate (cAMP) pathway is triggered promoting the translocation of cholesterol to the mitochondria. Subsequently, steroidogenesis is induced through the conversion of cholesterol into pregnenolone by the activity of cytochrome P450 11A1 (CYP11A1) (24, 25). Notably, stimulation of Leydig cells by LH is pivotal for the expression of proteins and enzymes involved with steroidogenesis ultimately leading to sustained production of testosterone.

Hormone therapy for prostate cancer patients specifically targets different aspects of this process. In clinical prostate cancer literature, androgen deprivation therapy (ADT) is generally used as a collective name for treatments that suppress androgen production and/or signaling. An overview of the androgen regulatory system and all the therapeutics that have been applied to prostate cancer patients since Huggins landmark study in 1941 is illustrated in Figure 1.

3. Evolution of androgen deprivation therapy

3.1. Surgical intervention and estrogen administration

After the initial study of Huggins and Hodges (2), gonadal androgen suppression by surgical or chemical castration was quickly introduced as a primary treatment for prostate cancer patients. While surgical intervention proved to increase survival substantially, patients eventually progressed. Huggins noted from earlier research that adrenal glands also contribute to androgen production in the human body and therefore conducted a study in 1945 removing the adrenal glands in multiple progressing castrated prostate cancer patients (26-29). However, due to the lack of available corticoid treatments, all patients suffered from high morbidity.

Shortly after, this approach was reiterated in light of novel therapeutics treating adrenal insufficiencies. However, the study yielded no significant clinical benefit other than reduction in sever bone aches (30). Two years later, another study was performed by Miller and Hinman, who aimed at chemically depleting adrenal testosterone through administration of high cortisone doses (31). Although >50% of the patients experienced positive effects, average response duration was relatively short (82 days). Furthermore, side-effects, such as lethargy and edema were highly prevalent. Three decades later, another study was performed treating patients with aminoglutethimide instead of cortisone (32). However, the results were similar to the study performed by Miller and Hinman.

Despite advantages, such as guaranteed depletion of gonadal testosterone and relatively low costs, medical castration was a more preferable choice than surgical castration due to the obvious reversibility features. To this end, estrogen administration was introduced together with surgical castration in the 1940s and was used in clinical practice until the 1980s. The most prescribed estrogen, diethylstilbestrol (DES), induces negative feedback on the hypothalamus-pituitary axis inhibiting LHRH and ultimately gonadal testosterone production. Although effects in patients were similar to surgical intervention, concerns were quickly raised by studies showing a strong association of estrogen administration and cardiac events in prostate cancer patients, especially in high doses of DES (33, 34). Lacking alternatives, estrogen administration for androgen deprivation was maintained for decades.

3.2. LHRH agonists and antagonists

In the 1980s, the landscape of advanced prostate cancer treatment was revolutionized by the introduction of LHRH agonists (35-40). Advantages compared to the previous estrogen

administration are the absence of potential cardiac events and their long-lasting effects, although surgical intervention remained the golden standard. LHRH agonists overstimulate the pituitary hormone LH initially resulting in a flare of testosterone production lasting approximately two weeks. In this period, an increase of tumor-related symptoms is often experienced by patients due to short-term overstimulation of the androgen receptor (AR) by high levels of testosterone. To relieve symptoms, treatment is often combined with a first generation AR inhibitor (bicalutamide, flutamide, cyproterone acetate) preventing increased binding of high androgen levels (41). Notably, no additional survival benefit has been reported in patients combining an LHRH agonist with a first generation AR inhibitor.

Continuing overstimulation of LHRH deregulates LH production within four weeks and indirectly depletes gonadal testosterone production. This effect is typically long lasting (up to three months) due to subcutaneous or intramuscular implantation of depots. While the morbidity level is low in patients treated with LHRH agonists, lack of sex drive and impotency are common. Furthermore, low testosterone levels in men are associated with osteoporosis risk and is therefore more prevalent in chemically castrated prostate cancer patients (42, 43). Today, physicians can choose from three different LHRH agonists, goserelin, leuprolide and triptorelin, of which goserelin and leuprorelin are most commonly prescribed. This is mainly due to the implant formulations being stable at room temperature, whereas triptorelin needs to be reconstituted before administration (44).

In the early 2000s, the LHRH antagonists, abarelix and degarelix, were introduced in Europe and the US (45). Some concerns were raised by the observation of testosterone microsurges during LHRH agonist treatment sparking the need for LHRH antagonists (46, 47). LHRH antagonists directly inhibit LH production, and therefore generally achieve gonadal testosterone depletion in a shorter period than LHRH agonists. Furthermore, in absence of a testosterone flare, no additional treatment with AR inhibitors is necessary. Studies have also demonstrated that abarelix also has properties to inhibit follicle stimulating hormone (FSH), which potentially contributes to the growth of prostate cancer (48-50). While these factors impose some advantages over LHRH agonists, prescription is limited in Europe and in the US the pharmaceutical has been withdrawn in 2006 due to disappointing sales and unforeseen allergic reactions.

3.3. AR inhibitors

Another treatment strategy that is used to manipulate AR signaling involves prevention of testosterone, and other androgens, binding the AR. AR inhibitors selectively bind the AR in the cytosol hindering potential activation and translocation to the nucleus. Hence, translation

of genes associated with cell growth and survival is inhibited. Early investigations in the 1960s and 1970s have led to the introduction of the first AR inhibitor to clinical practice in the 1990s (51, 52). Over the years multiple AR inhibitors were developed, which can be distinguished into two groups, steroidal and non-steroidal AR inhibitors. The main differences are the chemical structures, and more importantly, the side effects. Steroidal antiandrogens have progestational effects leading to prevalent decrease of sex drive and possible impotency. Nonsteroidal antiandrogens exclusively bind the AR and therefore lack the progestational side effects associated with steroidal antiandrogens.

Although one study showed that the steroidal AR inhibitor cyproterone acetate had similar clinical benefit and less observed side effects as the nonsteroidal AR inhibitor flutamide, steroidal AR inhibitors are currently not used as a primary treatment for prostate cancer patients (53, 54). Instead, first generation nonsteroidal AR inhibitors (bicalutamide and flutamide) are often combined LHRH agonists and antagonists to achieve a maximum androgen blockade. However, evidence of added clinical benefit is limited, which recently has led to the discontinuation of biculatumide treatment in Belgium (41). In other countries, bicalutamide is still combined with LHRH agonist treatment to relieve tumor-specific symptoms caused by the initial testosterone surge (41).

More recently, a second generation nonsteroidal inhibitor, enzalutamide, has been developed and introduced to clinical practice showing a substantial survival benefit in patients that are diagnosed with CRPC (55, 56). Notably, evidence of this survival benefit was also demonstrated hormone sensitive prostate cancer patients (HSPC) (57). Currently, other structural analogs of enzalutamide, darolutamide and apalutamide, are being investigated for their clinical efficacies (58, 59).

Studies that elaborate on testosterone levels in patient treated with AR inhibitors are scarce. However, some interesting observations were made in studies that incorporated patients treated with AR inhibitor monotherapy. Specifically, two studies in hormone naïve prostate cancer patients treated with bicalutamide or enzalutamide monotherapy showed that testosterone levels increased approximately 110% (60, 61). Notably, the underlying biochemical mechanisms are still unknown and therefore could be an interesting future research subject.

3.4. CYP17A1 inhibition

Despite increasing survival substantially by initial treatment with LHRH agonists or antagonists, patients inevitably progress to a castration resistant state. Various resistance

mechanisms have been revealed over the past years. One of the resistance mechanisms is the sensitization of the AR to low androgen levels being produced by the adrenal glands (62-64). Furthermore, previous studies also suggest that CRPC cells develop the capability of *de novo* androgen synthesis (65).

To target this resistance mechanism, testosterone production is completely depleted through combining LHRH agonist or antagonist treatment with an inhibitor of cytochrome P450 17A1 (CYP17A1) 17,20-lyase activity. This enzyme is responsible for converting the progestagens into androgens. Alternatively, the 21 hydroxylase activity of CYP17A1 converts progestagens into glucocorticoids and mineralocorticoids. To prevent development of congenital adrenal hyperplasia, glucocorticoids, such as prednisone, are often combined with CYP17A1 inhibition treatment. Although concerns were raised due to potential secondary glucocorticoid activation of the AR, an exploratory study did not find that baseline glucocorticoid levels were associated with a decrease in survival in metastatic prostate cancer patients (66).

The first CYP17A1 inhibitor that was applied for prostate cancer treatment was ketoconazole (67-70). Initially, ketoconazole was developed for fungal infections, although its effect on testosterone was quickly noted in a subset of men treated for fungal infections in an investigation of gynaecomastia (71). This off-label use of ketoconazole had the advantage of being cost-effective and the drug has low toxicities compared to chemotherapeutic drugs. Notably, ketoconazole induced a PSA response in 20-75 % of patients. However, no benefit in overall survival has been observed (72-78).

Three decades later, abiraterone was introduced as a new treatment option for metastatic CRPC patients, which, unlike ketoconazole, substantially improved overall survival (79). CYP17A1 inhibition by abiraterone is, in contrast to ketoconazole, not dose dependent and blocks the enzyme more effectively. This leads to increased anti-glucocorticoid effects, which could lead to adrenal inefficiencies. Therefore, abiraterone is always prescribed in combination with glucocorticoids (80-83). Similar to enzalutamide, the clinical benefit of abiraterone has also been demonstrated in HSPC patients (84, 85).

Data from two previous reports suggest that CYP17A1 inhibitors lower CYP17A1 inhibitors reportedly lower the testosterone concentration of castrated men approximately 20-fold (81, 83). Standard IA techniques are not capable of quantitating these levels, and even LC-MS/MS-based assays struggle with production of quantifiable results (80, 81). Until now, research exploring the clinical value of these levels has been scarce. One study investigated various androgens during abiraterone treatment and found that quantifiable levels of dehydroepiandrosterone were associated with a favourable progression-free survival (83).

Quantitation of testosterone remains difficult in these patients, which hinders studying testosterone as a prognostic biomarker for these patients.

3.5. Bipolar androgen therapy

Hormonal therapy focusing on lowering testosterone levels has been the backbone of advanced prostate cancer treatment for over 80 years. However, in the last decade, there has been a shift in perception regarding the effects of testosterone in CRPC. This change originates from articles describing protective properties against prostate cancer development and in patients that progressed after radical prostatectomy (86-90). The articles explain that, with the right timing and setting, testosterone stimulation can be beneficial for prostate cancer patients.

Notably, prior to this shift, preclinical models of metastatic CRPC already showed that administration of testosterone establishing supraphysiological levels resulted in apoptosis of cancer cells (91, 92). Later, it was demonstrated that prostate cancer cells adapt to the androgen-rich environment and continue growth after switching from testosterone ablation to testosterone stimulation (93). Importantly, these cells show resensitization to testosterone suppressive agents. Therefore, switching from testosterone ablation to testosterone stimulation, bipolar androgen therapy (BAT), together with consecutive testosterone ablation was suggested as a treatment for CRPC patients.

A pilot study performed in 2015 by Schweizer et al. showed that up to 3 cycles of switching was tolerated and 50% of patients had a PSA and radiographic response (94). Subsequently, a phase II trial conducted by Teply et al. demonstrated that 15 of 30 patients achieved a PSA response (> 50% decline) during enzalutamide treatment after androgen resensitization with BAT (95). Furthermore, only a small sample (10%) of patients suffered from hypertension during BAT. Notably, BAT was also found to be effective in a small cohort of patients in a hormone naïve state, for which it can be used to relieve patients of side-effects caused by long term testosterone suppression (96).

While testosterone plays a major role in BAT, the importance of monitoring of circulating testosterone levels is not yet underlined. Possibly, pharmacokinetic and pharmacodynamics parameters including serum testosterone could be investigated to further optimize this treatment strategy. Follow-up clinical trials are currently initiated that could contribute to this knowledge.

4. Testosterone analysis

4.1. Early testosterone analysis

Before standardized assays for testosterone analysis in blood were available, a methodology to assess effectiveness of chemical castration indirectly was proposed. Specifically, methods were developed for quantitation of steroid metabolites, generally 17-ketosteroid conjugates, which were extracted from patient urine (97-99). Notably, these assays were associated with laborious preanalytical procedures making them unsuitable for trials with high patient numbers. Still, various studies have been performed, albeit with low patient numbers, that demonstrated the association of steroid metabolites with surgical or chemically induced castration in prostate cancer patients (100-104). Although these studies found a short-term decrease in urinary metabolites after both surgical- and chemical castration, the concentrations recovered to their original levels after longer periods of time. Not surprisingly, other studies from that period demonstrated that steroid excretion had limited value as a predictor of testicular androgen production and circulating androgen levels (105, 106). Furthermore, Gallagher et al. emphasized that the contradictory results should be interpreted with scrutiny in their relation to castration efficacy (107).

The first method that allowed for quantitation of circulating testosterone levels was based on the fluorometric estimation of 17β-estradiol, which was yielded after enzymatic conversion of testosterone (108). However, this method was soon abandoned for double-isotope derivative assays that required substantially lower sample volumes and were associated with less laborious preanalytical procedures (109). The principle of the double-isotope derivative incorporated the addition of an isotopically labelled internal standard, either ^{14}C or deuterium labelled, and the formation of an isotopically labelled derivative in samples extracts, which was sometimes followed by the formation of a second derivative. Next, testosterone was purified by thin-layer and/or paper chromatography and finally, purified isotopically labelled testosterone was assayed by liquid scintillation counting.

Three studies incorporated this technique in order to investigate testosterone dynamics during castration therapy (3, 110, 111). Notably, all studies report pretreatment testosterone levels that are similar to levels found by modern assays in healthy individuals. Furthermore, in all castrated patients a significant and long-term drop in testosterone level is observed, albeit in strongly varying concentrations. While two studies report that most castrate levels of testosterone remain below the LLOQ of 1.7 nmol/L, another study reported a mean castrate level of 8.23 nmol/L. These findings indicate that double-isotope derivative assays lack 1)

sensitivity and 2) specificity in these concentration ranges, which was later confirmed by Wilke et al. (112). For these reasons, radioimmunoassays (RIA) able to quantitate plasma testosterone more efficiently were developed.

The notice that the competitive binding of an antibody could have quantitative properties for a specific antigen was first described in the landmark study performed by Berson and Yalow (113). The scientific community quickly realized that assays based on competitive antibody binding increased sensitivity and specificity substantially compared to double-isotope derivative assays and were particularly useful for sensitive quantitation of steroids (114). In addition, RIA generally had less laborious preanalytical procedures enabling routine measurements. Importantly, this enabled periodic monitoring of castration efficacy and therapy resistance. Furthermore, multiple hormones could be measured by separate RIA and included in trials.

First, these assays were applied to characterize low levels of steroid hormones in females (115). Later, studies focused on castrate levels of testosterone, especially those chemically induced by estrogens (4, 116, 117). The need for determining castration efficacy under estrogens, such as DES, was emphasized by concerns raised in a study published in 1967 by the Veterans Administration Cooperative Urological Research Group (VACURG) (33). Specifically, in patients treated with high doses of DES there was an increase in cardiovascular events observed, while no additional clinical benefit was evident. Therefore, it was hypothesized that low doses of DES were as efficient as higher doses and further evidence of adequate castration validated this claim.

Between the beginning of the 1980s and the 2000s, multiple studies were performed using RIA investigating the castration efficacy of LHRH agonists (46, 118-127) and one antagonist (128). Another study in that period investigated testosterone levels in surgically castrated prostate cancer patients (129). Notably, mean castration levels, as quantitated with RIA, of testosterone seem to have lowered over time. Especially studies after 1990 consistently report mean values below 1 nmol/L, which are concordant with values measured with contemporary assay techniques, i.e. automated IA and MS-based assays. A possible explanation could be the frequent application of extraction procedures after 1990. Previous studies have shown that purification procedures have proven to greatly enhance RIA accuracy (130). Unfortunately, most studies that were referred to lack detailed assay description necessary to assess this suggestion. Another explanation could be the lack of standardization between the assays. RIA kits were largely developed by small parties and not much effort was put in the standardization of assays between labs both nationally and internationally.

4.2. **Contemporary testosterone analysis**

4.2.1. Automated IA

IA technology was further optimized with the development of automated IA (131). In addition to improved specificity and sensitivity, automated analyzers were developed by large manufacturers that were equipped with short assay times. Furthermore, development of assays by large manufacturers also enabled standardization of assays on a broad scale.

Studies in castrated prostate cancer patients applying automated IA are listed in Table 1. This technology was first described for castrated prostate cancer patients by Sarosdy et al. (132). In this study, a long-lasting depot formulation for goserelin (10.8 mg every three months) was tested in 59 patients for 26 weeks using the ACS:180 analyzer (Bayer). Mean testosterone levels stayed below 1.0 nmol/L during the length of the study. These levels were comparable with one study using RIA for goserelin treated patients (123), and were lower than two other studies (124, 125).

Interestingly, three studies explicitly refuted the castration cut-off, which is based on levels measured with double-isotope derivative assays. A study performed by Oefelein et al. measured serum testosterone levels with an ACS:180 analyzer in 35 patients six months after orchiectomy procedures (5). Mean testosterone level was 0.52 nmol/L (Ranging from 0.17 – 1.0 nmol/L). These levels appear to be consistent with the testosterone levels measured by Sarosdy et al. (132), which used the same analyzer, although in that study patients were chemically castrated with the LHRH agonist goserelin. The levels were, as expected, substantially lower than the levels measured using double-isotope derivative assays (3, 110, 111). Subsequently, Morote et al. published two studies (Short term, 6 months; Long term, 3 years) using an identical group of 73 patients that were treated with LHRH agonists, in which the Immulite® 2500 was applied in both studies to quantitate serum testosterone levels (6, 133). The focus of these studies was to characterize breakthrough levels during LHRH agonist treatment and whether the occurrence of breakthrough levels could predict time to PSA progression and androgen independent progression. While mean testosterone levels remained below 0.69 nmol/L for all patients, some patients experienced breakthrough levels of testosterone (> 0.69 nmol/L) associated with poor prognosis. The testosterone levels were consistent with other studies performed applying automated IA and RIA (5, 46, 128, 129, 132), although the long-term study found some high concentrations (Up to 4.0 nmol/L).

Two other studies performed by Hara et al. measured testosterone levels with automated IA in castrated prostate patients (134, 135). Notably, patient samples were collected in the identical periods in the same hospital. However, the last study included one more patient, which probably resulted in the slightly different reported mean testosterone levels (Hara et al. 2012, 0.50 nmol/L vs. Hara et al. 2013, 0.52 nmol/L). While the author of both studies did not describe which analyzer was used in both studies, mean testosterone values seem similar to values observed by Oefelein et al. (5).

Clearly, standardized automated IA contributed greatly to the similarities of testosterone levels in castrated prostate cancer. All studies found comparable population intervals, which increases overall reliability of these assays. In addition to the advantages, also some limitations of automated IA are apparent. Automated testosterone automated IA notoriously lack specificity in low concentration ranges due to cross-reactivity with structural analogs resulting in potentially unreliable results (8-10, 15). Furthermore, sensitivity of these assays is often not adequate to accurately measure all castration levels. Manufacturers of automated IA addressed these issues in second generation assays. Notably, previous research has established that second generation testosterone assays have an improved accuracy in low concentration ranges compared to first generation testosterone assays (136, 137), although further improvement is still necessary. These limitations can be overcome using a specialized detection technique based on MS enabling enhanced sensitivity and specificity.

4.2.2. Mass spectrometry-based assays

Mass spectrometry (MS), either hyphenated with gas chromatography (GC) or with liquid chromatography (LC), has been used to measure steroids, especially testosterone, for decades. Due to the low volatility of steroids GC-based methods were mostly abandoned and replaced by LC-based assays capable of separating multiple steroids based on their polarity. Coupled with tandem-mass spectrometry (LC-MS/MS) instruments, testosterone assays achieved high specificity, also in low concentration ranges. Furthermore, over the recent years manufacturers enhanced sensitivity of their analyzers greatly and assays are highly customizable. In the latter case, choice of extraction procedure, addition of derivatization steps and application of two-dimensional (2D) LC can further enhance specificity and sensitivity of such assays, although caution should be taken with chemical derivatization due to possible formation of structural isomers (138).

Table 2 provides an overview of studies in castrated prostate cancer patients that applied LC-MS/MS-based methods. Notably, LC-MS/MS were only first described for their application in castrated prostate cancer patients until 2008. At this time, a novel hormonal therapy inhibiting the 17,20-hydroxylase activity of CYP17A1, abiraterone, was developed for CRPC patients, which also suppressed adrenal testosterone production. To differentiate between gonadal testosterone suppression alone and gonadal combined with adrenal testosterone suppression, ultra-sensitive LC-MS/MS were applied in multiple clinical studies investigating abiraterone efficacy (80-82, 139). A dose-finding study performed by Attard et al. using 21 patients and a follow-up for 145 days found that mean baseline testosterone levels (LHRH agonists alone) were 0.24 nmol/L (Range, < 1.2 nmol/L) and declined to undetectable levels (< 0.035 nmol/L) during abiraterone treatment in all patients. Similar levels were observed by Ryan et al. (mean, 0.017 nmol/L; SD, 0.005 nmol/L) and McKay et al. (Mean, 0.017 nmol/L; IQR, 0.010 – 0.021 nmol/L) in 1195 and 40 patients, respectively. Both studies had a follow-up period of 12 weeks. Notably, another study investigating 27 patients treated with abiraterone for 24 weeks estimated testosterone levels that were substantially higher (Mean, 0.23 nmol/L; Range, 0.017 – 0.49 nmol/L).

In addition, studies using LC-MS/MS assays were performed in patients that were treated with surgical or chemical castration. One study reported testosterone levels in 121 patients that transitioned from leuprorelin to degarelix administration (3 months follow-up) (140). During both treatments testosterone levels remained below the LLOQ (< 0.10 nmol/L). Conversely, Miyazawa et al. investigated testosterone levels in 36 patients that switched from degarelix to leuprorelin treatment and reported LC-MS/MS and automated IA results (141). Mean testosterone levels as measured with LC-MS/MS were 0.28 nmol/L (SD, 0.10 nmol/L). No significant differences with the automated IA results were observed. Another study investigated differences in testosterone levels between patients that were surgically castrated and chemically castrated with LHRH agonists (142). Paradoxically, the mean testosterone levels in the patients treated with LHRH agonists were significantly lower than the testosterone levels observed in patients that were surgically castrated. While no definitive conclusions could be made, the authors argued that due to absent gonadal testosterone LH levels are elevated in surgically castrated patients. This could activate LH receptors that have been identified in prostate cancer cells subsequently upregulating the expression of genes and enzymes involved with production of testosterone (143, 144). Lastly, two studies compared testosterone levels in patients treated with degarelix and LHRH agonists (145, 146). Separately, the studies found no significant differences between the two treatment groups. However, the overall concentrations reported by Sayyid et al. were approximately four times higher than the levels reported by Axcrona et al.

From these studies, it is clear that LC-MS/MS methods can achieve higher sensitivities than IA and could be capable of quantitation of nearly all testosterone levels found in castrated prostate cancer patients. Furthermore, studies in patients that are treated with abiraterone applying LC-MS/MS methods can differentiate these testosterone levels with castrate only levels. However, also some limitations are associated with LC-MS/MS-based testosterone methods. Not all LC-MS/MS methods achieved sufficient assay sensitivity to quantitate all castrate levels of testosterone and in similar treatment groups mean testosterone levels sometimes greatly differ between studies. This could be explained by LC-MS/MS assays being laboratory-developed and application of chemical derivatization to achieve additional sensitivity. Respectively, this can lead to a lack of standardization between methods and to a decrease in specificity due to isomer formation (13, 138). Importantly, these issues emphasize that more insights into the extent a specific LC-MS/MS-based assay is suitable to quantitate samples from castrated prostate cancer patients are needed, especially in relation to conventional automated IA.

5. Automated IA versus LC-MS/MS assays

Currently, testosterone levels of castrated prostate cancer patients are predominantly monitored by automated IA. The obvious advantages associated with automated IA, such as random-access and short turn-around times, are attractive for routine diagnostic labs. Furthermore, clinical laboratories often do not possess the resources or expertise to produce laboratory-developed LC-MS/MS assays. In addition, standardization protocols are more difficult to regulate for laboratory-developed assays.

Notably, in vitro diagnostic (IVD) MS systems suitable for standardized kits are available for routine diagnostics (147). This could be a solution for clinical laboratories struggling with development of homebrew-laboratory assays. However, IVD systems and kits are costly and the current selection in analyte is limited. Furthermore, current legislation allows for development of homebrew LC-MS/MS methods. For these reasons, it is still preferable for clinical labs to acquire non-IVD LC-MS/MS instruments to develop and validate a wide range of cost-efficient in-house assays.

Beside the practical aspects, it remains difficult to determine whether LC-MS/MS-based testosterone analysis is necessary for monitoring of castration efficacy in prostate cancer patients. For example, concentrations substantially lower than the recommended cut-off (< 1.7 nmol/L) are easily achieved using automated IA and LC-MS/MS, which would make LC-MS/MS analysis redundant according to current guidelines. However, in the context of this cut-off being based on double-isotope derivative assays, the adequacy of the cut-off is debatable. Notably, multiple studies have demonstrated that castrated testosterone levels remain at lower intervals and some studies already proposed a more appropriate cut-off at 0.69 nmol/L. Furthermore, recently published European guidelines emphasize that two other studies show that patients not breaking through a concentration of 0.69 nmol/L are associated with improved outcomes (133, 148).

A lower cut-off would change perspectives whether automated IA or LC-MS/MS would be more appropriate for determination of castrate levels of testosterone. Firstly, while all automated IA seem to have an LLOQ below the proposed cut-off (0.69 nmol/L), not all IA have a tested functional sensitivity (FS; imprecision below 20%) in these concentration ranges (See Table 3) (149, 150). Secondly, automated IA lack specificity around this cut-off and substantial discrepancies have been observed between automated IA and LC-MS/MS in women and children (14-16). Notably, these discrepancies are also observed in a regression and correlation analysis in healthy volunteer samples containing testosterone concentrations below 1.9 nmol/L (See Table 3) (149). Furthermore, our lab also observed differences up to

111% between a conventional automated IA (Roche Cobas instrument) and an in-house laboratory-developed LC-MS/MS assay in chemically castrated prostate cancer patients with or without addition of enzalutamide (See Figure 2). Therefore, a substantially biased result could lead to unnecessary change of treatment. In that regard, LC-MS/MS-based assays should be used as a reference when in doubt of adequate castration.

It remains debatable which value should be used to evaluate castration efficacy. As LC-MS/MS-based analysis remains the golden standard, reference intervals in castrated prostate cancer patients determined by this technique could be used to establish an accurate cut-off. However, the lack of standardization between laboratory-developed LC-MS/MS assays hinders the definition of a universal cut-off. To account for inter-assay variability, we therefore recommend that castrate testosterone reference intervals should be determined for all separate LC-MS/MS-based assays that are used for monitoring testosterone in prostate cancer patients or should be harmonized with another testosterone LC-MS/MS assay that has properly established such reference intervals. In addition, a study comparing testosterone analysis in castrated prostate cancer patients by multiple conventional IA and a standardized LC-MS/MS assay should be performed to evaluate the suitability of automated IA to monitor adequate castration and determine the appropriate castration cut-off.

6. Potential clinical utility of ultra-sensitive testosterone assays

In addition to monitoring adequate castration, castrate levels of testosterone could potentially be used as a prognostic or predictive biomarker for prostate cancer treatments. It is hypothesized that high residual testosterone levels originating from the adrenal gland or produced within the tumor microenvironment could attribute to increased stimulation of the AR, free from androgen suppression by LHRH agonists or antagonists, that trigger tumor growth. To this end, Ryan et al. previously performed a study in 1195 CRPC patients either treated with abiraterone plus prednisone or placebo plus prednisone showing that low baseline testosterone levels (< 0.080 nmol/L) were associated with significantly poorer outcomes than higher baseline testosterone levels (≥ 0.30 nmol/L) (151). In more recent reports, Ryan et al. evaluated the predictive value of baseline testosterone levels in 1050 CRPC patients treated with the chemotherapeutic agent docetaxel (152, 153). Median overall survival in low versus high baseline levels of testosterone was not significantly different. However, a decline in testosterone levels during treatment was associated with improved overall survival, albeit with an HR of 1.02.

Two other studies have found similar results, although they are mostly underpowered, retrospectively designed and apply automated IA. The first study investigated a cut-off (0.174 nmol/L) at baseline before LHRH agonists or antagonists either plus enzalutamide (n = 35) or docetaxel (n = 38) (154). While no significant differences in overall survival were observed, high baseline testosterone levels (> 0.174 nmol/L) were associated with favourable and unfavourable median progression-free survival for enzalutamide treated patients and docetaxel treated patients, respectively. The second study applied the same cut-off (0.174 nmol/L) in patients treated with LHRH agonists or antagonists either plus abiraterone (n = 43) or enzalutamide (n = 72). For both treatment groups, significant differences in median progression-free survival and overall survival were observed in favour of high baseline testosterone levels (> 0.174 nmol/L).

Although these reports seem promising, testosterone analysis has not yet been implemented in clinical practice for these patients. Probably, this is due to some uncertainties that have not been addressed. Firstly, baseline measurements of testosterone are a snapshot of a patient's testosterone level during treatment. Low individuality of castrate levels of testosterone, i.e. intra-individual levels range over the whole population interval, could make correct interpretation of these measurements difficult. To gain insight into the within- and between-patient biological variation of testosterone in these patients, a biological variation study according to guidelines from the European Federation of Clinical and Laboratory Medicine (EFLM) Working Group should be performed (155). Secondly, treatment of

advanced prostate cancer is changing rapidly. First line CRPC treatments, such as abiraterone and enzalutamide, have been proven to be effective in the treatment of HSPC (57, 85). Furthermore, new hormone therapies (apalutamide, darolutamide and bipolar androgen therapy) are currently being investigated in clinical trials for which studies evaluating the prognostic or predictive value of baseline testosterone levels should be conducted (58, 59, 95). Lastly, large prospective trials applying periodical LC-MS/MS testosterone measurements at baseline and weeks after initiation of treatment using a predefined cut-off should be performed to validate current evidence of testosterone as a biomarker in prostate cancer patients.

These uncertainties pose a significant challenge. However, it should be emphasized that there is an important unmet need for biomarkers in HSPC and CRPC patients. While most prostate cancer patients benefit substantially from hormone therapy, a small percentage does not respond to treatment or immediately demonstrate progressive disease. In addition, with the availability and development of new therapeutics for CRPC patients it is difficult to choose the right treatment for the right patient. Ultimately, further investigation into the prognostic and predictive value of testosterone in relation to hormone therapy in prostate cancer patients could address this issue.

7. Conclusion

Over the decades and at least in the near future, testosterone analysis continues to play a central role in the management of prostate cancer patients treated with hormonal therapies. Development and application of increasingly sensitive and accurate techniques have enabled more adequate evaluation of castration efficacy. Furthermore, contemporary LC-MS/MS-based testosterone assays are able to quantitate nearly all levels found in surgically and chemically castrated prostate cancer patients. Based on the findings presented in this review, some clinical and research recommendations are suggested that are presented in Table 4 to respectively i) improve the current practice of testosterone analysis in prostate cancer, or ii) to further improve the understanding and clinical utility of ultra-sensitive testosterone assays for prostate cancer patients.

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Table 1. Publications reporting castrate levels of testosterone using automated IA.

Ns = Not specified

Author	Year	Analyzer	Assay LLOQ (nmol/L)	Therapy	Patients - nr.	Follow-up	Mean testosterone - nmol/L (SD/95%CI/Range)
Sarosdy et al.	1999	ACS:180	0.35	Goserelin	59	26 weeks	< 1.0
Oefelein et al.	2000	ACS:180	0.35	Orchiectomy	35	ns	0.52 (Range, 0.17 - 1.0)
Morote et al.	2007	Immulite® 2500	0.52	LHRH agonist	73	6 months	< 0.69 (ns)
Morote et al.	2009	Immulite® 2500	0.52	LHRH agonist	73	3 years	< 0.69 (Range, < 4.0)
Hara et al.	2012	ns	0.17	Goserelin	71	6 months	0.5 (SD, ± 0.3)
Hara et al.	2013	ns	0.17	Goserelin	72	6 months	0.52 (SD ± 0.35)

Table 2. Publications reporting castrate levels of testosterone using LC-MS/MS assays.

Ns = Not specified

Author	Year	Analyzer	Derivatization	Assay LLOQ (nmol/L)	Therapy	Patients -nr.	Mean testosterone - nmol/L
de la Rosette et al.	2011	Ns	Ns	0.1	Leuprolerin and degarelix	121	< 0.1 (ns)
van der Sluis et al.	2012	Quattro Premier™ XE	Yes, methoxylamine	0.1	Orchiectomy or LHRH agonist	66	LHRH agonists, 0.13 (Range, < 0.70); Orchiectomy, 0.32 (Range, < 0.98)
Axcrona et al.	2012	Ns	Ns	ns	Degarelix and goserelin	201	0.17 (ns)
Miyazawa et al.	2015	Ns	Ns	ns	Leuprorelin	36	0.28 (SD, ± 0.10)
Sayyid et al.	2017	Quattro Premier™ XE	Yes, hydroxylamine	ns	Degarelix or LHRH agonist	39	Degarelix, 0.69 (Range, 0.0 - 1.6); LHRH agonist, 0.63 (Range, 0.0 -1.1)

Table 3. FS (LLOQ as determined by La'ulu et al.)**, manufacturer specified sensitivity parameters and pearson correlation coefficients derived from a method comparison with an LC-MS/MS method.** FS, correlation coefficients, intercepts, slopes and mean biases were determined by La'ulu et al. LLOQ and LOD were specified by Montagna et al. Three separate testosterone LC-MS/MS assays were used in the two referenced works and in the generation of our own data.

	Technical Specifications (IA)			Method comparison with LC-MS/MS (<1.9 nmol/L) [149]				
	FS - nmol/L [149]	LLOQ - nmol/L [150]	LOD - nmol/L [150]	n	Intercept - nmol/L (95%CI)	Slope (95%CI)	*r*	Mean Bias - % (95%CI)
Abbott ARCHITECT	< 0.14	0.08	0.05	124	0.02 (-0.15 to 0.18)	1.14 (0.79 to 1.48)	0.73	19.2 (-47.5 to 85.8)
Beckman Coulter DxI	0.36	NA	0.35	124	-0.08 (-0.46 to 0.30)	2.01 (1.53 to 2.49)	0.66	63.8 (-12.0 to 139.6)
Roche Cobas	NA	0.416	0.087	58	-0.26 (-0.49 to -0.085)*	1.96 (1.16 to 3.05)*	0.71*	0.79 (-109.3 to 110.8)*
Siemens Immulite 2000	3.49	0.5	NA	124	-0.18 (-1.21 to 0.85)	1.31 (0.61 to 2.01)	0.42	14.2 (-57.9 to 86.4)
* As defined for a method comparison in our lab using 58 castrated prostate cancer patients								

Table 4. Clinical and research recommendations for interpretation of testosterone results, establishing accurate castration cut-off values and application of testosterone as a clinical biomarker.

Category	Issue	Cause	Recommendation
Clinical	Reliability of unusually high IA testosterone levels during chemical castration treatment	Automated IA lack specificity due to cross-reactivity in concentration ranges found in castrated prostate cancer patients	When in doubt of an IA generated testosterone result, reassess with an adequate LC-MS/MS assay
Clinical	Appropriateness of the testosterone castration cut-off	Current testosterone cut-off recommended in clinical practice guidelines is based on 50-year old double-isotope derivative assays. More recent data indicate that a lower cut-off is more appropriate, although adequate assay sensitivity and specificity is lacking	Determine castrate testosterone reference intervals for each laboratory-developed LC-MS/MS assay or Ias not harmonized with an LC-MS/MS assays that has properly established such reference intervals
Clinical	Required measurement range for clinical LC-MS/MS assays used to determine castration testosterone levels	Testosterone LC-MS/MS assays can have various measurement ranges	Based on levels observed in Figure 2 and intervals reported by studies incorporated in this review, the LLOQ of an LC-MS/MS based assay for testosterone should be at least 0.05 nmol/L to enable testosterone quantitation in castrated men
Research	Adequacy of IA to monitor castrate levels of testosterone	Automated IA lack specificity due to cross-reactivity in concentration ranges found in castrated prostate cancer patients	Compare multiple conventional automated IA with a standardized LC-MS/MS assay using castrate prostate cancer patient samples
Research	High intra-patient variability of castrate testosterone levels could prohibit its application as a biomarker	Hormones are characterized by their high intra-individual variability	The within- and between-patient variation should be estimated to evaluate the individuality of castrate levels of testosterone
Research	Prognostic or predictive value of castrate testosterone levels for prostate cancer treatments	Some evidence has been reported that baseline testosterone levels are prognostic for abiraterone treatment outcome, although sufficient evidence on other CRPC treatments and its predictive value is lacking	Prospective trials with predefined cut-off values should be performed to validate evidence

Figure 1. Overview of testosterone regulation, hormonal therapeutics and their targets.

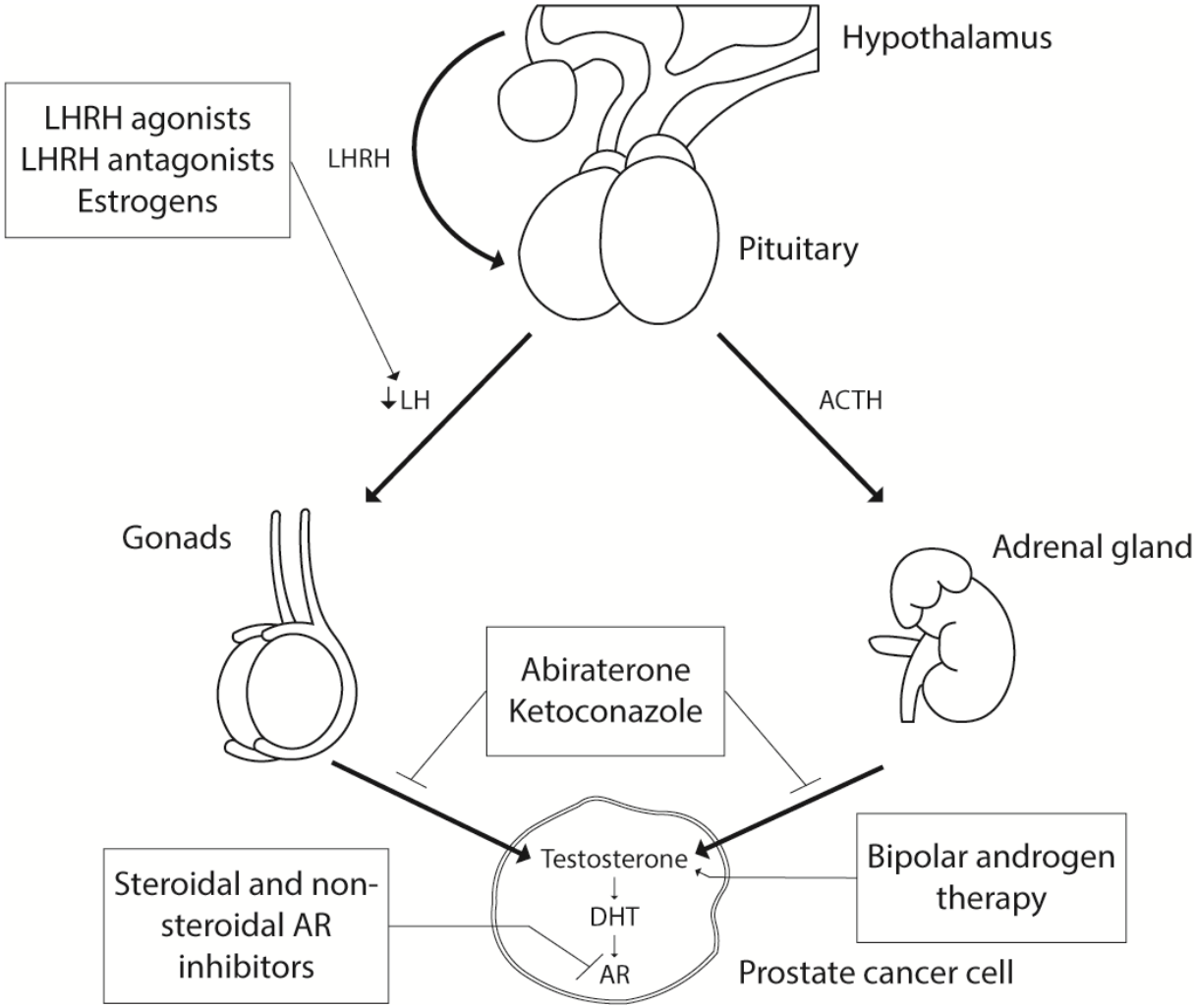
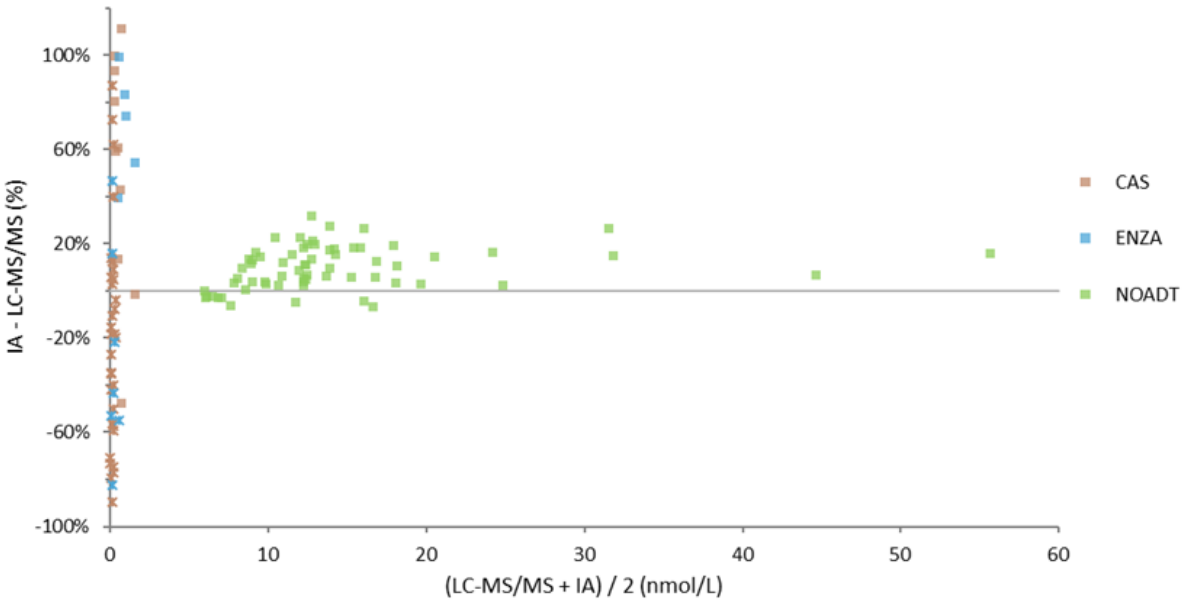


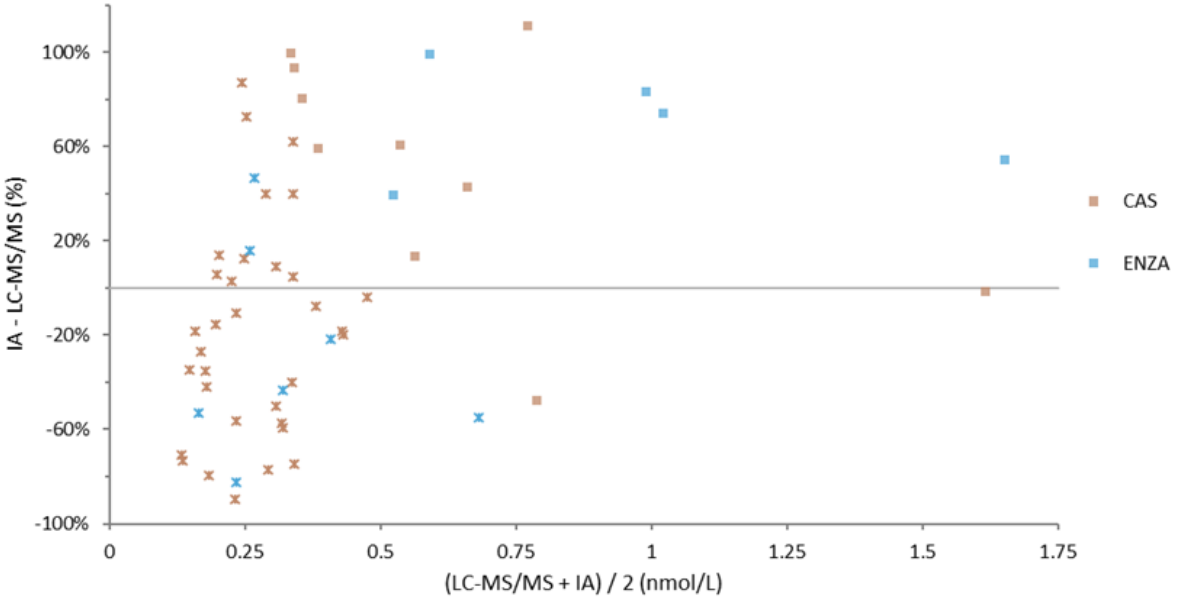
Figure 2. Relative difference plots of the method comparison between the newly developed LC-MS/MS assay and the 2nd generation testosterone assay on a Cobas E601/602 system. (A) shows all samples that were quantifiable with both methods (n = 120), whereas (B) shows samples that were quantifiable of patients receiving CAS and ENZA (n = 55).

CAS = chemical castration only
ENZA = chemical castration with enzalutamide
NOADT = without ADT
LC-MS/MS = liquid chromatography tandem-mass spectrometry
IA = immunoassay

A


100%
60%
20%
-20%
-60%
-100%
IA - LC-MS/MS (%)
0
10
20
30
40
50
60
(LC-MS/MS + IA) / 2 (nmol/L)
CAS
ENZA
NOADT

B


100%
60%
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-100%
IA - LC-MS/MS (%)
0
0.25
0.5
0.75
1
1.25
1.5
1.75
(LC-MS/MS + IA) / 2 (nmol/L)
CAS
ENZA

Chapter 2.2

Testosterone analysis in castrated prostate cancer patients: suitability of the castration cut-off and analytical accuracy of the present-day clinical immunoassays

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Abbreviations:

ADT	androgen deprivation therapy
AIA	automated immunoassay
CI	confidence interval
IQR	interquartile range
LC-MS/MS	liquid chromatography tandem –mass spectrometry
LHRH	luteinizing hormone-releasing hormone
LLOQ	lower limit of quantitation
LOD	limit of detection
Nr.	number
PCa	prostate cancer
TE	total allowable error

Abstract

Objectives: Testosterone testing is relevant for evaluating castration adequacy and diagnosis of castration-resistant prostate cancer (PCa). However, the recommended testosterone cut-off of 1.7 nmol/L (50 ng/dL) to define adequate castration is based on consensus and not validated for the automated immunoassays (AIA) used in today's medical laboratories. Furthermore, appropriate population intervals have not been determined by a state-of-the-art assay. We investigated the analytical suitability of this cut-off and the accuracy of the present-day AIAs for testosterone analysis in castrated PCa patients.

Methods: Leftover serum from 120 PCa patients castrated with luteinizing hormone-releasing hormone agonists was analysed for testosterone by five methods: Architect i2000 (Abbott), Access (Beckman), Cobas 6000 (Roche), Atellica (Siemens), LC-MS/MS. For all assays, the castration 95th, 97.5th and 99th percentile upper limits were determined. Furthermore, Passing-Bablok regression, mean bias and Spearman's correlation coefficients were compared to the LC-MS/MS method and total error based on biological variation.

Results: All castration upper limits, ranging from 0.472 nmol/L (LC-MS/MS) to 1.25 nmol/L (Access) (95% percentile), were significantly lower than the current castration cut-off (1.7 nmol/L). Slopes of Passing-Bablok regressions comparing the AIA with the LC-MS/MS method ranged from 1.4 (Cobas and Atellica) to 3.8 (Access). The Architect showed the highest correlation with LC-MS/MS (ρ=0.58). All AIA failed to meet the desirable total error criterion.

Conclusions: These results suggest that a lower general testosterone castration cut-off may be more appropriate in evaluating the adequacy of castration in PCa and that present-day AIA lack analytical accuracy to quantify testosterone levels in castrated PCa.

Key words: hormone sensitive prostate cancer, chemical castration, testosterone, population interval, immunoassay, liquid chromatography tandem-mass spectrometry (LC-MS/MS)

Introduction

Monitoring testosterone levels in prostate cancer (PCa) patients treated with androgen deprivation therapy (ADT) is important in assessing adequacy of castration treatment and diagnosis of castration resistant PCa (1). To this end, a testosterone cut-off of 1.7 nmol/L (50 ng/dL) has been defined by clinical guidelines as adequate castration criterion (2, 3). Notably, this cut-off is based on consensus and a clinical validation study investigating response duration (4). However, for the latter the type of testosterone assays applied was not specified.

The 1.7 nmol/L castration cut-off probably origins from historical testosterone assays that are no longer applied in today's clinical laboratory practice (5). Furthermore, it is generally accepted that these historical assays are inferior to the state-of-the-art method, liquid chromatography tandem-mass spectrometry (LC-MS/MS), in terms of analytical sensitivity, specificity and accuracy (6). However, due to the costs and the complexity of developing and operating LC-MS/MS-based testosterone assays in routine clinical laboratory practice predominantly automated immunoassays (AIA) are applied to determine testosterone concentrations.

Although several previous studies in females and children have demonstrated that these assays are particularly inaccurate at low testosterone concentrations (< 1.9 nmol/L) and correlate poorly with LC-MS/MS-based assays (7-10), similar analyses have not been published for castrated PCa patients. Furthermore, no appropriate testosterone population intervals have been determined for this patient population. To investigate whether the castration cut-off value of 1.7 nmol/L is still valid as a criterion for adequate castration, we have determined upper limits of the testosterone population interval for a best-practice testosterone LC-MS/MS-assay and four clinically applied testosterone AIA based on a cohort of castrated PCa patients. In addition, we compared the testosterone concentrations obtained by AIAs to the LC-MS/MS method and evaluated the analytical suitability of their accuracy for testosterone quantitation in this population.

Materials and methods

Samples

Consecutive leftover serum samples from castrated PCa patients were collected between March 2018 and March 2021. Blood collection occurred between 7:30 am and 5:00 pm. A total sample size of 120 patients was obtained after exclusion as recommended by Clinical and Laboratory Standards Institute guidelines (11). PCa patients were eligible when treated

with a luteinizing hormone-releasing hormone (LHRH) agonist and excluded if 1) they received androgen receptor (AR) inhibitors, CYP17A1 inhibitors or chemotherapeutic agents, and 2) blood collection occurred within three months after the first LHRH agonist depot injection due to possible occurrence of an treatment-induced testosterone flare. Prior to the start of our study, approval of the Institutional Review Board was obtained (IRBd18-145). The study is in accordance with the declaration of Helsinki.

Testosterone measurements

Testosterone concentrations were determined in duplicate using a LC-MS/MS-based testosterone assay, routinely applied at the Netherlands Cancer Institute (12, 13). This assay was standardized against the SRM971 reference standard (National Institute of Standards and Technology, Gaithersburg, MD, United States). Furthermore, testosterone measurements were performed on the next four AIA: Roche Cobas® 6000 and Cobas® Pro (2^{nd} generation testosterone assay), Abbott Architect i2000SR (2^{nd} generation testosterone assay), Beckman-Coulter DxI 600 Access and Siemens Atellica® IM 1300 (2^{nd} generation testosterone assay). Due to limited sample volume, testosterone concentrations determined by AIA were measured in singular.

Data analysis

A nonparametric method was used to determine testosterone population intervals (11). Herein, right-sided 95^{th}, 97.5^{th} and 99^{th} upper limit percentiles and their 90% confidence intervals (CI) were calculated to represent assay-specific upper limits of the population intervals. To test whether testosterone levels were significantly different between each assay, pairwise Wilcoxon tests were performed. In pairwise comparisons, p-values were adjusted with the Bonferroni method. Agreement between AIA and LC-MS/MS was assessed with Passing-Bablok regressions and Spearman's correlation coefficients. Differences in slopes and intercept were considered statistically significant if the 95%CI did not include 1 and 0, respectively. Bias relative to the state-of-the-art method (LC-MS/MS) and between the four AIA was investigated using relative difference plots. Mean bias was defined as the mean relative difference and was compared to the total error (TE) as determined in healthy volunteers (16.5%) and castrated PCa patients (24.8%) (12, 14). Differences in mean bias were considered statistically significant if the 95%CI did not include the maximum allowable TE. Population intervals and Wilcoxon tests were performed with R (Version 4.0.2). Passing-Bablok regressions and difference plots were generated using Analyse-it (Version 5.90).

Results

Of the 129 samples, nine were excluded based on exclusion criteria. Patient characteristics of the remaining 120 samples are shown in Table 1. Testosterone levels as measured for each assay are shown in Figure 1 and descriptives, assay-specific lower limit of quantitation (LLOQ), limit of detection (LOD) and population intervals are listed in Table 2. Three samples contained insufficient volume for measurement with the Abbott AIA. For all AIA, the testosterone results were highly different from the LC-MS/MS assay ($p<0.0001$). For the Abbott, Beckman, Roche and Siemens assays, 1 (1%), 29 (24%), 106 (88%) and 26 (22%) of testosterone results were below the manufacturer lower limit of quantitation (LLOQ), respectively. For the LC-MS/MS, none of the testosterone results were below the LLOQ. The upper population interval limits of the AIA ranged from 0.66 to 1.25 nmol/L and were all substantially higher than upper population interval limit of the LC-MS/MS assay.

In Figure 2, Figure 3 and Table 3 Passing-Bablok regressions, relative difference plots and correlations between AIA and the LC-MS/MS are displayed. Comparisons between AIA are shown in Supplementary Material, Figures 1 and 2. Passing-Bablok regressions in Figure 2 were generated using testosterone concentrations equal to and above the LLOQ of both assays except for comparisons with the Beckman assay, for which only a LOD is specified in the manufacturer's manual. For comparison between the AIA and the LC-MS/MS (Figure 2, Figure 3, Table 3), all regression slopes positively deviated from 1 (ranging 1.4 to 3.8), of which only the Roche AIA was not significantly different. Intercepts ranged from -0.35 to 0.16 and all AIA except the Beckman AIA were significantly different from 0. Mean bias of the AIA compared to the LC-MS/MS ranged from 90.5% (Siemens) to 168% (Beckman), which was substantially higher than the maximum allowable TE of 24.8%. In addition, the AIA showed Spearman's correlation coefficients between 0.15 (Roche) and 0.58 (Abbott). Results were similar for Abbott and Siemens compared to the LC-MS/MS in samples equal to and above the LOD. For Roche, the Passing-Bablok slope was 2.1 (95%CI, 1.5 to 3.0), the intercept was negative (95%CI, -0.31, -0.60 to -0.15) and correlation improved ($\rho=0.28$). In the inter-AIA comparison (Supplementary Material Figures 1 and 2; Table 3), Passing-Bablok regression slopes (0.31 to 2.2) and intercepts (-0.54 to 0.32) were variable for all samples. Mean bias ranged from -14.0% to 67.7% and Spearman's correlation coefficients ranged from 0.23 to 0.76.

Discussion

The first aim of the current study was to determine the testosterone population interval for chemically castrated men and their corresponding upper population limits using a sensitive

LC-MS/MS-based assay and four clinically applied AIA. For the LC-MS/MS-based assay, we calculated upper limits at 0.472, 0.495 and 0.579 nmol/L for the 95th, 97th and 99th percentiles, respectively. Furthermore, the testosterone concentrations and the upper limits determined by LC-MS/MS were significantly lower than obtained for the AIA. Also, all obtained AIA castration upper limits for testosterone were substantially lower than the castration cut-off of 1.7 nmol/L. These findings suggest that the choice of testosterone assay greatly influences the observed testosterone level in castrated PCa patients and indicates that a lower castration cut-off seems appropriate. The results are in line with previous studies reporting testosterone concentrations measured by LC-MS/MS in castrated PCa patients (15, 16), although one study found testosterone levels similar to those found by AIA (17). It should be noted that the latter study did not report on standardization and quantitated testosterone using hydroxylamine derivatization, which could lead to formation of cis- and trans isomers and falsely elevated results (18).

In a second analysis, this study aimed to evaluate the analytical performance of four testosterone AIA clinically applied to quantitate testosterone concentrations in castrated men. The AIA testosterone results were compared with LC-MS/MS using Passing-Bablok regressions, Spearman's correlation coefficients and relative difference plots. Mean relative differences were compared with the desirable TE as determined in healthy volunteers and castrated PCa patients. All slopes significantly deviated from 1, except for the Roche AIA. This is explained by the low number of quantifiable samples of the Roche AIA leading to broad 95%CI range of the slope (0.58 to 4.8) and low correlation (ρ=0.15). For other AIA, the highest correlation was achieved with the Abbott AIA (ρ=0.58), which still can be categorized as poor. Mean relative differences were all substantially higher than the desirable TE derived from PCa patients. These results suggest that in terms of bias and correlation with LC-MS/MS, clinically applied AIA, or at least the four evaluated in this study, are analytically unsuitable for testosterone quantitation in castrated men. Our findings align with the varying testosterone levels reported in previous studies investigating chemically castrated PCa patients (5), as well as the lack of between-AIA correlation observed in women and children (7, 10).

In addition, AIA results equal to and above the LOD and between-AIA comparisons were investigated. Although from an analytical perspective not acceptable, values equal to and above the LOD have been included in clinical validation studies of steroid hormones in cancer patients (19). When using all results equal to and above the LOD in the method comparison study, this particularly influenced regression parameters (slope, 2.1 v. 1.4; intercept, -0.31 v. 0.16), correlation (0.28 v. 0.15) and mean relative difference (20.1% v.

132.2%) for the Roche AIA in comparison with LC-MS/MS. This can be explained by a substantial increase in quantifiable samples when including samples with concentration between the LOD and LLOQ (48% v. 12%). Although mean relative difference was within desirable TE limits, the slope of the Passing-Bablok regression exceeded the TE criterion and correlation was very limited. In addition, between-AIA comparisons were investigated. Between-AIA comparison showed variable results in Passing-Bablok regression, correlation and mean relative difference. None of the comparisons revealed acceptable characteristics.

The population interval variation raises the question whether a general testosterone cut-off is appropriate for evaluating adequacy of castration. The LC-MS/MS-based assay indicates that adequately castrated PCa patients have testosterone concentrations that are at least approximately three times lower. In the same patient samples, AIA results appear to reach higher levels. However, taking into account cross-reactivity by structurally related compounds and other factors known to compromise the AIA accuracy at these low concentrations, it is very likely that these testosterone results do not represent actual testosterone concentrations (20, 21). This is confirmed by the lower testosterone concentrations determined by the LC-MS/MS assay known to be less sensitive for such interferences. The limited analytical accuracy of AIA also indicates that when doubt arises about the accuracy of a castrated testosterone level, and especially if this result has major treatment implications, one should consider testosterone analysis by LC-MS/MS-based assays. Furthermore, when low testosterone concentrations are investigated as a prognostic or predictive marker, for example in castrated prostate cancer patients, adequate assay specifications are essential in enabling a proper assessment of the study results (22). Based on the presented results and inaccuracy of the investigated AIAs, testosterone concentrations in clinical studies have to be determined by LC-MS/MS measurement systems with an appropriate LLOQ (5). Another issue is choosing which criterion to use in determining castration population levels. By definition, reference intervals are the 95% interval of all observations. Since, in this context of castration, only an upper limit or cut-off is relevant, we specified both the 95% and 97.5% interval. Notably, since for some clinical indications a higher test specificity is desirable, e.g. for the troponin cut-off used to exclude myocardial infarction (23), we also calculated the 99% interval. When using any of these criteria, the testosterone castration cut-off remains significantly lower than 1.7 nmol/L for all investigated assays. Therefore, allowing testosterone castration concentrations up to 1.7 nmol/L might result in sub-optimal PCa treatment.

Some limitations were associated with this study. Firstly, patients were retrospectively included, which could have introduced selection bias. Secondly, in this study, we did not

control for different types of LHRH agonists (24), treatment with LHRH antagonists and surgical castration (25). However, due to irreversibility, surgical castration is rarely performed for prostate cancer nowadays. Furthermore, it should be noted that, to the best of our knowledge, no studies applying an LC-MS/MS assay that report differences between LHRH agonist types and between LHRH antagonists and LHRH agonists have been published.

Although the limits in sensitivity and specificity of testosterone AIA have been underlined in literature (7, 8, 10), they are still applied in clinical studies, in which testosterone is related to primary study endpoints for castrated PCa patients (4, 26, 27). Here, we demonstrate that, in these patients, highly variable testosterone results are generated by four clinically applied AIA, which could greatly affect and bias study outcomes. In addition, we show that the upper limits of the population interval determined by LC-MS/MS were substantially lower than the currently applied castration cut-off. These results indicate that, in evaluating adequacy of castration in PCa patients, a lower castration cut-off may be more appropriate than the currently applied 1.7 nmol/L.

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Table 1. Patient characteristics.

Abbreviations

IQR = interquartile range

Nr. = number

Due to rounding off, percentages may not add to 100%.

	Patients (n=120)
Age - yr (IQR)	73 (68-76)
LHRH Agonist - nr (%)	
Goserelin	95 (79)
Leuprorelin	20 (17)
Triptorelin	5 (4)
Gleason Score - nr (%)	
6-8	75 (63)
9-10	38 (32)
Unknown	7 (6)
Metastases - nr (%)	
Yes	111 (93)
No	9 (7)
PSA - ng/mL (IQR)	2.3 (0.081-10)
ALP - (U/I) (IQR)	86.6 (75.0-112)

Table 2. Testosterone population intervals in prostate cancer patients stratified by assay.

	Testosterone (nmol/L)				
Parameter	**LC-MS/MS (n=120)**	**Abbott Archtitect i2000SR (n=117)**	**Beckman DxI 600 Access (n=120)**	**Roche Cobas® 6000/Pro (n=120)**	**Siemens Atellica® IM 1300 (n=120)**
Limit of detection (LOD)	NAa	0.05	0.35	0.087	0.17
Samples below LOD, nr. (%)	0	0 (0)	29 (24)	62 (52)	25 (21)
Lower limit of quantitation (LLOQ)	0.025	0.15	NAb	0.416	0.24
Samples below LLOQ, nr. (%)	0	1 (1)	NAb	106 (88)	26 (22)
Median	0.220	0.430	0.420	< 0.416	0.400
Mean	0.210	0.426	0.490	< 0.416	0.373
Upper limit - 95th percentile (90%CI)	0.472 (0.466 - 0.595)	0.911 (0.822 - 1.06)[c]	1.25 (1.24 - 1.35)	0.660 (0.609 - 1.06)	0.700 (0.700 - 0.900)
Upper limit - 97.5th percentile (90%CI)	0.495 (0.466 - 0.595)	1.02 (0.610 - 1.15)[c]	1.32 (1.24 - 1.35)	0.911 (0.609 - 1.06)	0.800 (0.700 - 0.900)
Upper limit - 99th percentile (90%CI)	0.579 (0.466 - 0.595)	1.40 (1.38 - 1.81)[c]	1.35 (1.24 - 1.35)	1.03 (0.609 - 1.06)	0.900 (0.700 - 0.900)

SI conversion factors: To convert testosterone to ng/dL, multiply values by 28.8184.

[a] LOD was not determined for the LC-MS/MS assay

[b] No LOD is specified for the Beckman assay

[c] Due to a sample size below 120, 90%CI of the reference limits were calculated by bootstrapping.

Abbreviations

LC-MS/MS = Liquid chromatography tandem-mass spectrometry

LLOQ = Lower limit of quantitation

Nr = Number

Table 3. Intercept, slope, Spearman's rho and mean bias for each analytical method combination.

Abbreviations

LLOQ Lower limit of quantitation

		≥LOD					≥LLOQ				
Method 1	Method 2	n	Intercept - nmol/L (95%CI)	Slope (95%CI)	ρ	Mean bias, % (95%CI)	n	Intercept - nmol/L (95%CI)	Slope (95%CI)	ρ	Mean bias, % (95%CI)
Reference method comparison											
LC-MS/MS	Roche Cobas® 6000/Pro	58	-0.31 (-0.60 to -0.15)	2.1 (1.5 to 3.0)[a]	0.28	20.1 (-5.9 to 46.2)	14	0.16 (-1.1 to 0.36)	1.4 (0.58 to 4.8)	0.15	123 (36.8 to 210)[b]
LC-MS/MS	Abbott Archtitect i2000SR	117	-0.022 (-0.11 to 0.054)[a]	2.2 (1.8 to 2.6)[a]	0.59	121 (103 to138)[b]	116	-0.022 (-0.11 to 0.055)	2.2 (1.7 to 2.6)[a]	0.58	120 (102 to 138)[b]
LC-MS/MS	Beckman DxI 600 Access	91	0.35 (-1.1 to -0.044)[a]	3.8 (2.7 to 6.3)[a]	0.26	168 (126 to 211)[b]	NA[c]	NA[c]	NA[c]	NA[c]	NA[c]
LC-MS/MS	Siemens Atellica® IM 1300	95	0.074 (-0.0069 to 0.16)	1.5 (1.1 to 1.7)[a]	0.53	89.8 (75.5 to 104)[b]	94	0.086 (0.0083 to 0.16)[a]	1.4 (1.1 to 1.8)[a]	0.52	90.5 (76.0 to 105)[b]
Between-AIA comparison											
Roche Cobas® 6000/Pro	Abbott Archtitect i2000SR	58	0.21 (0.047 to 0.30)[a]	1.3 (0.96 to 1.7)	0.45	62.0 (49.9 to 74.0)[b]	14	-0.35 (-5.1 to 0.080)	1.9 (1.2 to 11.4)[a]	0.24	18.8 (-5.10 to 42.7)
Roche Cobas® 6000/Pro	Beckman DxI 600 Access	54	0.0025 (-0.47 to 0.20)	2.2 (1.5 to 4.0)[a]	0.52	67.7 (53.1 to 82.3)[b]	14[d]	-0.22 (-3.4 to 0.51)	1.7 (0.80 to 8.7)	0.54	37.1 (19.6 to 54.5)
Roche Cobas® 6000/Pro	Siemens Atellica® IM 1300	57	0.32 (0.27 to 0.35)[a]	0.62 (0.51 to 0.78)[a]	0.72	55.9 (45.6 to 66.2)[b]	14	0.31 (0.12 to 0.50)[a]	0.62 (0.24 to 0.84)[a]	0.76	6.70 (-4.40 to 5.20)
Abbott Archtitect i2000SR	Beckman DxI 600 Access	88	-0.54 (-0.99 to -0.23)[a]	2.2 (1.7 to 3.3)[a]	0.23	7.90 (-5.20 to 20.9)	83[d]	-0.39 (-0.91 to -0.12)[a]	2.0 (1.4 to 2.9)[a]	0.23	7.90 (-5.20 to 20.9)
Abbott Archtitect i2000SR	Siemens Atellica® IM 1300	92	0.13 (0.069 to 0.20)[a]	0.63 (0.50 to 0.77)[a]	0.57	-6.40 (-13.1 to 0.400)	91	0.15 (0.077 to 0.20)[a]	0.61 (0.50 to 0.77)[a]	0.55	-6.60 (-13.4 to 0.200)
Beckman DxI 600 Access	Siemens Atellica® IM 1300	84	0.29 (0.23 to 0.36)[a]	0.31 (0.22 to 0.40)[a]	0.43	-14.0 (-25.0 to -5.50)	66[d]	0.26 (0.13 to 0.37)[a]	0.35 (0.21 to 0.50)[a]	0.43	-14.0 (-25.0 to -3.00)

[a] Intercept and slope are significantly different from 0 and 1, respectively

[b] Mean bias and 95%CI are outside TE boundaries

[c] No LOD is specified for the Beckman assay and all patients have LC-MS/MS testosterone levels above its LLOQ

[d] The LOD of the Beckman assay was used for this comparison

Figure 1. Testosterone levels measured per assay.

Assay LLOQ and median values are displayed as horizontal black solid and dashed lines, respectively. Asterisks (*) are used to indicate p-values (*, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001). Testosterone AIA are abbreviated to the manufacturers name.

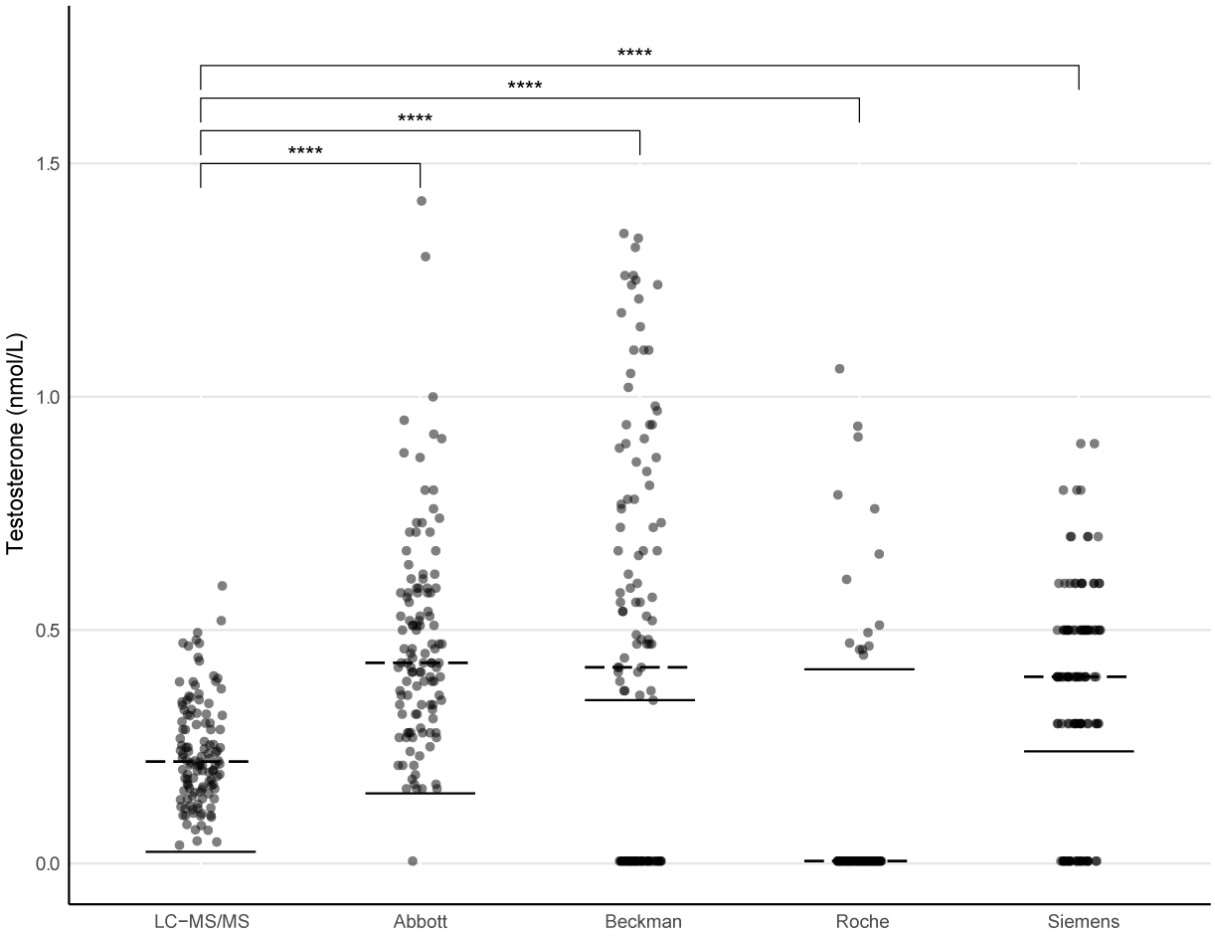


Figure 2. Scatterplots and Passing-Bablok regression lines (black) for comparisons between the four AIA and the LC-MS/MS assay.

Concentrations equal to and above the LLOQ are displayed with circle symbols and concentrations between the LOD and LLOQ are displayed with triangle symbols. Testosterone AIA are abbreviated to the manufacturers name.

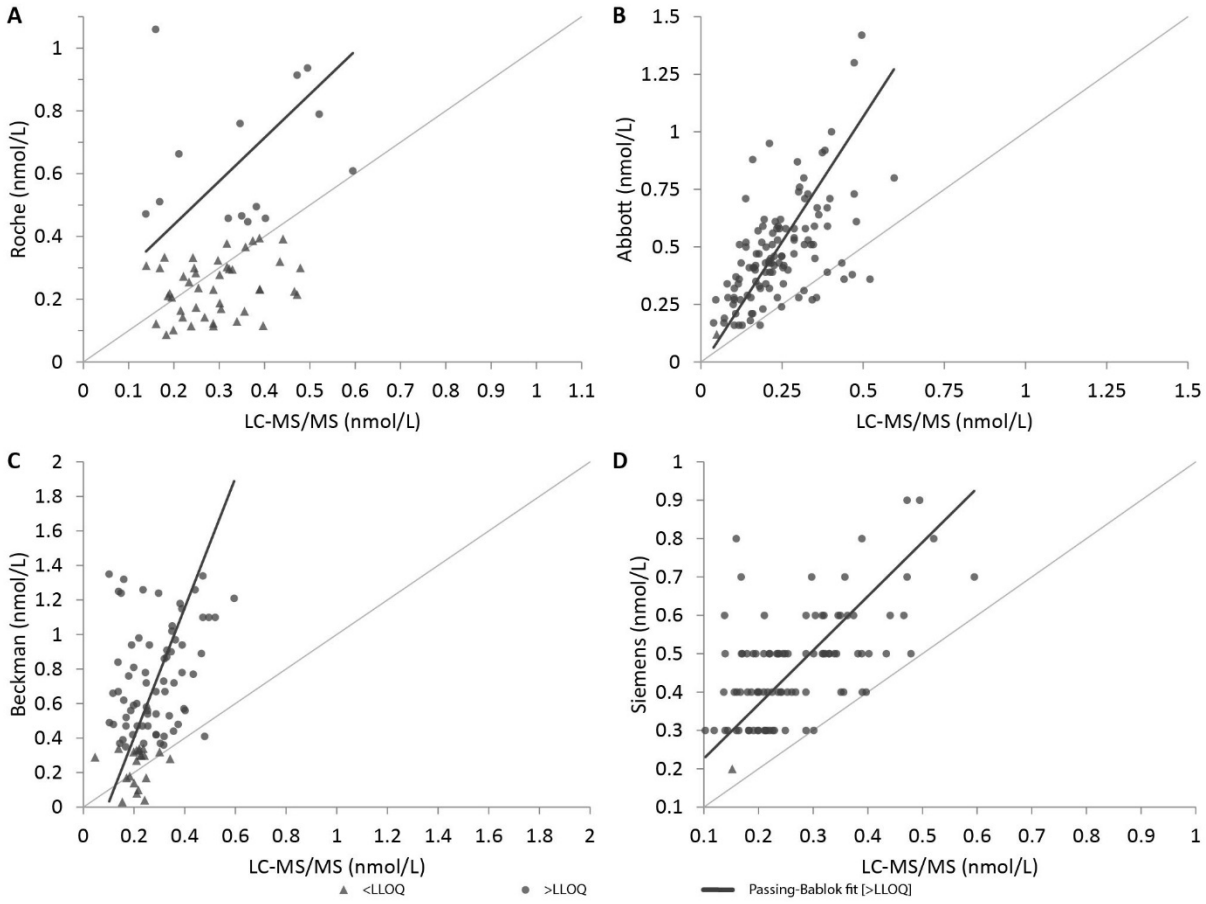
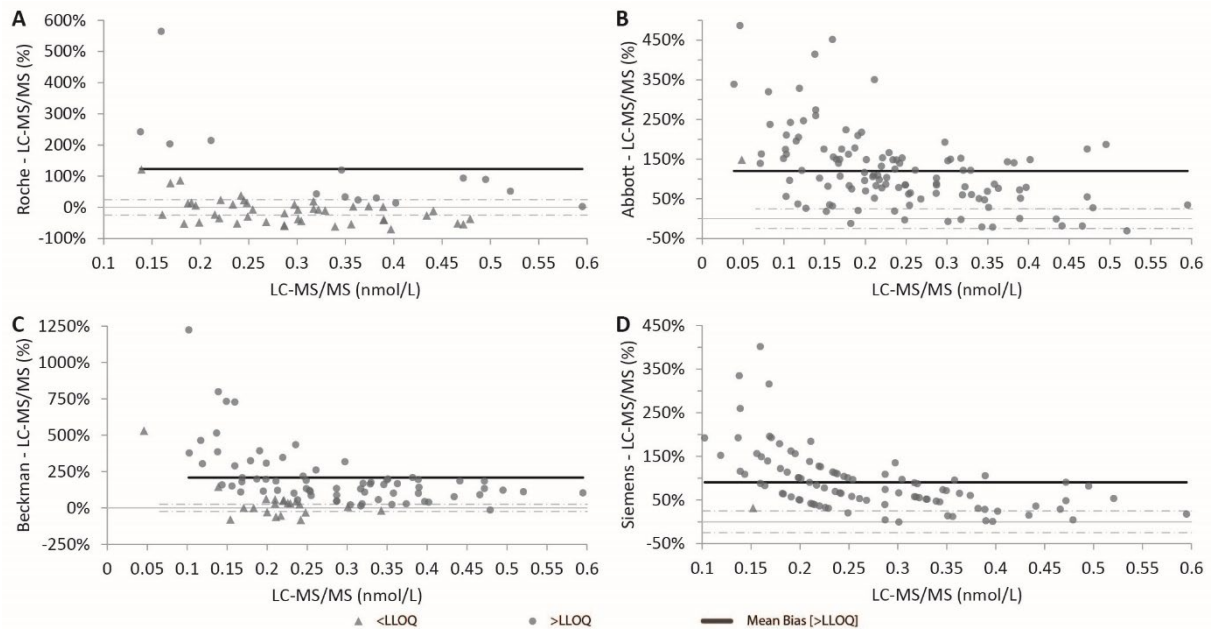


Figure 3. Relative difference plots for comparisons between the four AIA and the LC-MS/MS assay.

LC-MS/MS concentrations are displayed on the x-axis. Concentrations equal to and above the LLOQ are displayed with circle symbols and concentrations between the LOD and LLOQ are displayed with triangle symbols. Testosterone AIA are abbreviated to the manufacturers name.



Chapter 2.3

Serum testosterone measured by liquid chromatography-tandem mass spectrometry is an independent predictor of response to castration in metastatic hormone-sensitive prostate cancer

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Running title: Testosterone levels and progression-free survival in metastatic hormone-sensitive prostate cancer patients

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Word count: 2501
Tables: 2
Figures: 3
Supplemental Material: Yes

Key words: serum testosterone, hormone-sensitve prostate cancer, LC-MS/MS, survival, castration

Abbreviations

ALP	alkaline phosphatase
CI	confidence interval
CRPC	castration-resistant prostate cancer
IA	immunoassay
HR	hazard ratio
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LHRH	luteinizing-hormone releasing-hormone
mHSPC	metastatic hormone-sensitive prostate cancer
PFS	progression-free survival
PSA	prostate-specific antigen

Abstract

Background: Although testosterone levels have been associated with progression-free survival (PFS) in metastatic hormone-sensitive prostate cancer (mHSPC) patients, this has primarily been investigated using inaccurate immunoassays (IA). In the present study, we investigated whether castrate testosterone levels determined by a liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay is an independent risk factor for treatment response in mHSPC.

Methods: In total, 106 mHSPC patients treated with luteinizing-hormone releasing-hormone (LHRH) agonists were retrospectively analyzed between March 2018 and August 2021. Testosterone levels in serum samples were quantitated using an LC-MS/MS assay. In a subset of patients, IA (Roche Cobas Pro) values were compared with LC-MS/MS results. Association of these risk factors with PFS was estimated using the Kaplan-Meier product limit method and Cox proportional hazard models.

Results: Median PFS was shorter for high testosterone levels (>0.231 nmol/L, 18.4 v. 42.6 months, HR 1.7, p=0.018), PSA responses above 4 ng/mL (13.8 v. 38.9 months, HR 2.7, p<0.0001) and high tumor volume (20.9 v. 38.9 months, HR 1.8, p = 0.018). Low testosterone levels and a PSA response below 4 ng/mL was associated with longer median PFS (46.2 months) than the remaining combinations (13.8-19.3 months, HR 3.4 - 5.8, overall p<0.01). In 67 patients, testosterone levels below the median remained associated with longer PFS, whereas IA measurements did not show a similar difference.

Conclusion: Our results suggest that, in addition to PSA response and tumor volume, high testosterone levels measured by LC-MS/MS during chemical castration is an independent response predictor for mHSPC patients.

Introduction

The discovery that androgens function as an important driver in prostate cancer by Charles Huggins marked a long-lasting role for androgen suppression in the systemic treatment of prostate cancer patients (1). Nowadays, metastatic hormone-sensitive prostate cancer (mHSPC) is treated with luteinizing hormone-releasing hormone (LHRH) agonists or antagonists (2). These agents suppress gonadal androgen production through interaction with the hypothalamic-pituitary-gonadal axis (3-5). Circulating levels of testosterone are monitored during LHRH agonist or antagonist treatment to assess castration adequacy (2, 6, 7). Although international guidelines have defined a castration cut-off at 1.7 nmol/L, this cut-off is based on assays (eg. Immunoassays, IA) not suitable for accurate quantitaion of low concentration ranges (8). Notably, in a recent study, we found that four commonly applied testosterone IA produced significantly different results from a best-practice liquid chromatography-tandem mass spectrometry assay and eachother in samples from 120 castrated mHSPC patients (9). It is likely that these discrepancies are caused by a lack of analyte specificity and method inaccuracy at low testosterone concentrations in IA, which could have implications for the clinical application of the current castration cut-off.

In the present study, we aimed to investigate the relationship between testosterone levels quantitated by a clinically applied LC-MS/MS and progression-free survival (PFS) in men with mHSPC. In addition, in a subset of patients, the association with PFS of either testosterone results generated by LC-MS/MS or a commonly applied IA was compared.

Methods

Patients and study design

Patients diagnosed with mHSPC that had their serum testosterone levels measured by LC-MS/MS at the request of the treating urologist during visits at the Antoni van Leeuwenhoek hospital between March 2018 and August 2021 were eligible. Patients were excluded based on 1) objection to scientific research, 2) blood collection within 1 month after initial chemical castration, 3) additional treatment with androgen receptor targeted agents or chemotherapy during chemical castration. Approval was obtained from the institutional review board (IRBd18145) and the study followed the guidelines set by the declaration of Helsinki.

Testosterone analysis

Blood was collected from mHSPC patients during chemical castration. Following blood withdrawal, serum was obtained using Rapid Serum Tubes (BD, Franklin Lakes, NJ, United States) and stored either one week at 4 °C or up to one year at – 20 °C. For all samples,

serum testosterone was quantitated using an LC-MS/MS assay available at the Antoni van Leeuwenhoek hospital (10, 11). Briefly, testosterone was extracted with liquid-liquid extraction and LC-MS/MS analysis consisted of reversed phase chromatography and consecutive multiple reaction monitoring. The LC-MS/MS assay was able to accurately quantitate serum testosterone levels as low as 0.025 nmol/L. IA testosterone results were obtained using a Cobas 6000/Pro (Roche, 2nd generation testosterone assay) analyzer.

Data analysis and statistics

For survival analysis, time to castration-resistant prostate cancer (CRPC) was back-calculated to the start of chemical castration treatment. CRPC diagnosis, or PFS, was defined according to the recommendations published by the Prostate Cancer Clinical Trials Working group 3, i.e. either PSA progression (two consecutive increases of 25% and 2 ng/mL above the nadir) or radiographic progression while testosterone levels remained below 1.7 nmol/L (12). Patients were censored when 1) no event had occurred at the final follow-up moment or 2) a patient was lost to follow-up. Time to CRPC for different serum testosterone groups (low and high according to a cut-off at the median value) of the first during treatment sample, low and high tumor burden and PSA responses below 4 ng/mL either alone or as a combination of two factors was investigated using Kaplan Meier curves. In addition, if available, multiple testosterone LC-MS/MS and IA measurements were included during treatment to 1) generate median, minimum and maximum within-patient testosterone concentrations and 2) compare prediction of time to CRPC between a commonly applied IA and an LC-MS/MS method. Differences in median time to CRPC were tested using the long-rank test. To further investigate the relationship between these risk factors and time to CRPC, univariate Cox proportional hazards models were generated. A multivariate Cox proportional hazard model was established adjusting for, in case of univariate significance, age, Gleason score, baseline PSA and alkaline phosphatase (ALP).

Nonparametric Mann-Whitney U tests were used to assess differences in continuous variables and chi-squared tests were used to assess differences in categorical variables. Hazards ratios (HR) and their confidence intervals (CI) were extracted from Cox regressions. An alpha of 0.05 was applied for all hypothesis tests. No correction was applied for multiple testing. All statistical analyses in the present study were programmed and executed in R (Version 3.6.3).

Results

Based on the predetermined criteria, 106 of 298 eligible patients were included for further investigation (See Supplementary Figure 1). The median testosterone concentration was

0.231 nmol/L. Median time between initiation of chemical castration and collection of the first serum sample was 280 (IQR 104 – 608) days. Patient characteristics are listed in Table 1. In the low testosterone group, more patients had PSA responses below 4 ng/mL ($p = 0.012$) and baseline PSA was higher in the high testosterone group ($p = 0.05$). Median follow-up was 21.4 months. All measured testosterone concentrations were below 1.0 nmol/L.

In Figure 1, Kaplan-Meier curves according to testosterone groups (low and high), PSA response below 4 ng/mL and tumor volume are displayed. Median PFS was shorter for high testosterone levels compared with low testosterone levels (18.4 v. 42.6 months, $p = 0.018$, Figure 1A) at first during treatment testosterone measurement. Similar results were observed for median, minimum and maximum within-patient testosterone values (Supplementary Figure 2). Patients with PSA responses below 4 ng/mL had a longer median PFS than patients with PSA responses remaining above 4 ng/mL (13.8 v. 38.9 months, $p < 0.0001$). Patients with high tumor volume had shorter median PFS compared with the low tumor volume group (20.9 v. 38.9 months, $p = 0.018$). In addition, from 67 patients IA measurements were obtained for comparison (Figure 2). In only 12 of these 67 samples testosterone could be quantitated above the lower limit of quantitation (LLOQ, 0.416 nmol/L). Applying the LLOQ as cut-off did not reveal a difference between median PFS ($p = 0.18$), whereas patients with high testosterone levels, as measured with the LC-MS/MS method (> median), had shorter times to CRPC ($p < 0.01$).

Next, we investigated differences in time to CRPC for combinations of testosterone groups, PSA response and tumor volume (Figure 3). A longer time to CRPC was found for patients with low testosterone levels and a PSA response below 4 ng/mL upon chemical castration (46.2 months) compared to patients with one or both risk factors (13.8 - 19.3 months, overall $p < 0.001$). Patients with low testosterone levels and low tumor volume median PFS was longer (42.6 months) than for patients with high testosterone levels and high tumor volume (12.5 months, overall $p < 0.01$). Patients with either high testosterone levels or high tumor volume had median PFS of 21.4 and 24.7 months, respectively. A similar trend was observed for the combination of PSA response and tumor volume (Supplementary Figure 3).

In a final analysis, our data was used to generate Cox proportional hazard models (Table 2). Analysis of individual risk factors showed that high testosterone levels (HR 1.7, 95%CI 1.1 - 2.8, $p = 0.02$), PSA responses above 4 ng/mL (HR 2.7, 95%CI 1.6 - 4.5, $p < 0.001$) and high tumor volume (HR 1.8, 95%CI 1.1 - 3.0, $p = 0.02$) were associated with a shorter time to CRPC. Univariate analysis of testosterone levels as a continuous variable were in line with these results (HR 20.9, 95%CI 3.4 – 130, $p < 0.01$). In multivariate analysis of individual risk factors, Cox proportional hazard models were adjusted for localized prostate cancer

treatment and baseline PSA levels. After adjustment, HR for CRPC development 1) increased for the high testosterone group (HR 3.3, 95%CI 1.5 – 7.2, p < 0.01), 2) was similar for PSA responses above 4 ng/mL (HR 2.6, 95%CI 1.2 – 5.6, p = 0.01) and 3) decreased for tumor volume (HR 1.5, 95%CI 0.67 – 3.2, p = 0.34) compared to univariate analysis. In an additional analysis, individual risk factors were combined and analyzed in Cox proportional hazard models. In univariate analysis, testosterone groups with PSA responses showed that high testosterone levels and/or PSA responses above 4 ng/mL were associated with a shorter time to CRPC (HR 3.4 - 5.8, p < 0.01). Similarly, high testosterone levels and a high tumor volume were associated with a shorter time to CRPC (HR 1.8-3.4, p<0.01). Multivariate analysis revealed an increased HR for testosterone groups combined with PSA response (HR 4.0 – 8.2, p < 0.0001) and testosterone groups combined with tumor volume (2.4 – 6.6, p < 0.001).

Discussion

In this study, the predictive value of serum testosterone as measured by LC-MS/MS was investigated for the prediction of CRPC development in men with castration for prostate cancer. Testosterone results from the first during treatment samples were grouped as low or high based on a cut-off at the median population value (0.231 nmol/L). Patients with high testosterone levels showed shorter times to CRPC (HR 1.7, 95%CI 1.1 - 2.8, p = 0.02) and including significant risk factors as baseline PSA and localized treatment in a multivariate model further increased the predictive value (HR 3.3, 95%CI 1.5 – 7.2, p < 0.01). This indicates that correcting for localized treatment and PSA at baseline strengthens the relationship between testosterone levels and risk of progression. Notably, a similar association was not found for testosterone results that were generated by a commonly applied IA. These results suggest that testosterone levels, generated by LC-MS/MS analysis, in addition to PSA response and tumor volume, might be used to identify patients that will progress to castration-resistance early.

Previous studies have already described the relationship between castrate testosterone levels and survival (13, 14). Notably, these reports typically refer to substantially higher testosterone levels (> 0.7 nmol/L and > 1.7 nmol/L) than those observed in our LC-MS/MS cohort. This can be explained by the application of IA for the quantitation of testosterone levels, which are notoriously inaccurate at low concentration ranges (15). Our lab recently performed a method comparison between four routinely applied IA and one best-practice assay using serum samples from 120 chemically castrated mHSPC patients (9). In this analysis, the testosterone results quantified by IA were significantly higher than those quantified by the LC-MS/MS assay, and none of the IA was consistent with the LC-MS/MS

assay, highlighting the technical shortcomings of IA in evaluating castration adequacy in mHSPC patients. Notably, this explains the high castration cut-offs found in previous studies.

In a follow-up analysis, testosterone groups were combined with either PSA response upon chemical castration (below 4 ng/mL) and tumor volume, which are identified prognostic parameters for castration treatment response (16-18). Patients with low serum testosterone levels upon chemical castration and a PSA response below 4 ng/mL had an improved response to treatment compared to patients with high testosterone levels and/or PSA response above 4 ng/mL (HR 4.0 – 8.2, $p < 0.0001$). Furthermore, patients with low testosterone levels and low tumor volume had an improved PFS over patients with high testosterone levels and/or high tumor volume (2.4 – 6.6, $p < 0.001$). While these results indicate that these risk factors are complementary and independent, the differences between individual strata are not clear-cut. Therefore, further investigation in larger patient populations is warranted, for example, in a secondary analysis of the ongoing large randomized-controlled trials.

It should be noted that this study describes results from a cohort not including patients with contemporary standard of care. Recently, following long term results of the ongoing STAMPEDE, CHAARTED, LATITUDE and GETUG-AFU15 trials, upfront docetaxel or abiraterone treatment combined with ADT was implemented as standard of care for mHSPC patients (16, 18-20). Combinations with other AR targeted agents, such as enzalutamide, apalutamide, darolutamide and abiraterone, were investigated in the ENZAMET, ARCHES, PEACE-1, ARASENS and TITAN trials. Importantly, results show a benefit of double and even triple AR targeting in the early castration treatment of prostate cancer (21-25). Notably, the improvement in survival of the intervention arms of these studies could be explained by the additional AR targeting in patients with high testosterone levels. Therefore, accurate testosterone measurement by LC-MS/MS might aid in identifying a subset of patients particularly benefiting from additional, more toxic, hormonal therapy in mHSPC.

In recent years, tumor volume has been investigated as a risk factor for the first-line treatment of mHSPC patients leading to its application in standard clinical care (16-18). This risk factor has an advantage over classical prostate cancer risk factors, such as Gleason score, baseline PSA and ALP levels, due to its direct relation with disease burden. However, classification of tumor volume by radiologists can be somewhat difficult in cases that are not apparent. Furthermore, differences in results were observed between the STAMPEDE, CHAARTED and GETUG-AFU15 trials indicating variability in the application of this risk factor. Although information on this risk factor can be collected at baseline, combination with

other risk factors, such as PSA response or castrate testosterone levels (26, 27), could improve results.

Our study also has some apparent limitations. Firstly, clinical data was analyzed retrospectively and patients were included based on serum testosterone requests made by the treating physicians, which could have led to selection bias. For example, patients not responding well to chemical castrations treatment could be more likely to have their testosterone levels examined. Notably, median PFS observed in this study is higher than observed in previous studies (28, 29). The higher median PFS could be explained by the inclusion of patients with long responses prior to sample collection. An analysis of 62 patients that had blood sampling within one year after LHRH agonist treatment initiation revealed median PFS that was in line with previous studies and a similar difference between testosterone groups. Secondly, grouping of patients could be influenced by a high variation in testosterone levels. However, in a previous study, we demonstrated that within-patient castrate testosterone levels are stable relative to population range (30). Finally, serum was collected at variable times during the day. Circadian regulation of testosterone might have influenced our results, although the magnitude of this effect in castrated men is unknown and we did not observe a correlation between testosterone levels and hour of day (Data not shown).

While previous studies have associated testosterone levels with time to disease progression (13, 14), they commonly applied inaccurate IA at low concentration ranges, generally found in castrated men. Furthermore, these studies mostly lacked a specification of the testosterone method used, while our lab recently demonstrated significant differences between four commonly applied IA and an LC-MS/MS assay (9). In this study, our results suggested that, in addition to established risk factors PSA response (≤ 4 ng/mL) and tumor volume, testosterone levels determined by an accurate best-practice LC-MS/MS-based method above the median (> 0.231 nmol/L), but not by IA, might be used as an independent risk factor for mHSPC treatment. Furthermore, our results indicated that testosterone levels, PSA response and tumor volume were to some degree complementary. To further optimize treatment of metastatic mHSPC patients, castration testosterone levels, as measured by LC-MS/MS, should be evaluated to guide treatment decisions in larger prospective clinical trials

Acknowledgements

None.

Funding

No external funding was received for this study.

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Table 1. Patient characterization.

Abbreviations:

ALP = alkaline posphatase

PSA = prostate-specific antigen.

Characteristic	Testosterone		p-value	overall p-value
	≤ 0.231 nmol/L	> 0.231 nmol/L		
No. of patients	53	53		
Age, yr - mean (SD)	70 (7)	69 (8)	0.3	
Gleason Score - n (%)*				
6-7	30 (57)	20 (38)		
8-10	18 (34)	30 (57)		0.064
Unknown	5 (9)	3 (6)		
Tumor volume - n (%)*				
High	18 (34)	19 (36)		
Low	31 (58)	31 (58)		1
Unknown	4 (8)	3 (6)		
PSA below 4 ng/mL - n (%)*				
Yes	41 (77)	30 (57)		
No	8 (15)	21 (40)		0.012
Unknown	4 (8)	2 (4)		
Local treatment - n (%)*				
Radical prostatectomy	23 (43)	22 (42)		
Radiotherapy	7 (13)	9 (17)		
Active surveillance	2 (4)	0 (0)		0.51
High intensity focused ultrasound	0 (0)	1 (2)		
Metastasis at PCa diagnosis	21 (40)	21 (40)		
PSA, ng/mL - median (IQR)	10.1 (5.2-27.8)	16.7 (8.2-57.0)	0.05	
Testosterone, nmol/L - median (IQR) **	0.173 (0.138-0.196)	0.301 (0.267-0.360)	<0.001	
ALP, U/l - median (IQR)	80 (69-102)	82 (65-116)	0.7	

* Percentage sums may deviate from 100 due to rounding off

** Testosterone was quantitated using an ultrasensitive LC-MS/MS assay

Table 2. Cox proportional harzards analysis for progression-free survival.

Abbreviations:

PSA = Prostate-specific antigen

		Univariate		Multivariate	
	Events/at risk	HR (95%CI)	p value	HR (95%CI)	p value
Testosterone					
≤0.231 nmol/L	32/53	1.0 (ref)	0.02	1.0 (ref)	<0.01
>0.231 nmol/L	39/53	1.7 (1.1-2.8)		3.3 (1.5-7.2)	
PSA below 4 ng/mL*					
Yes	43/71	1.0 (ref)	<0.001	1.0 (ref)	0.01
No	24/29	2.7 (1.6-4.5)		2.6 (1.2-5.6)	
Tumor volume**					
High	27/37	1.8 (1.1-3.0)	0.02	1.5 (0.67-3.2)	0.34
Low	39/62	1.0 (ref)		1.0 (ref)	
Testosterone; PSA below 4 ng/mL*					
≤0.231 nmol/L; Yes	21/41	1.0 (ref)	<0.01	1.0 (ref)	<0.0001
≤0.231 nmol/L; No	7/8	3.4 (0.91-13.0)		4.0 (1.0-16)	
>0.231 nmol/L; Yes	22/30	3.9 (1.3-11)		4.3 (1.5-13)	
>0.231 nmol/L; No	17/21	5.8 (1.8-18)		8.2 (2.6-26)	
Testosterone; Tumor volume**					
≤0.231 nmol/L; Low	18/31	1.0 (ref)	<0.01	1.0 (ref)	<0.001
≤0.231 nmol/L; High	11/18	1.8 (0.83-3.9)		2.4 (0.68-8.4)	
>0.231 nmol/L; Low	21/31	1.9 (0.97-3.5)		4.6 (1.4-15)	
>0.231 nmol/L; High	16/19	3.4 (1.7-6.8)		6.6 (1.7-25)	
PSA below 4 ng/mL; Tumor volume					
Yes; Low	25/43	1.0 (ref)	<0.01	1.0 (ref)	<0.001
Yes; High	15/24	1.8 (0.95-3.6)		1.8 (0.62-5.0)	
No; Low	11/15	3.1 (1.5-6.6)		3.8 (1.3-11)	
No; High	12/13	3.5 (1.7-7.2)		3.2 (1.1-9.5)	

* From 6 patiens no baseline PSA levels were available

** From 7 patients information on tumor volume was not available

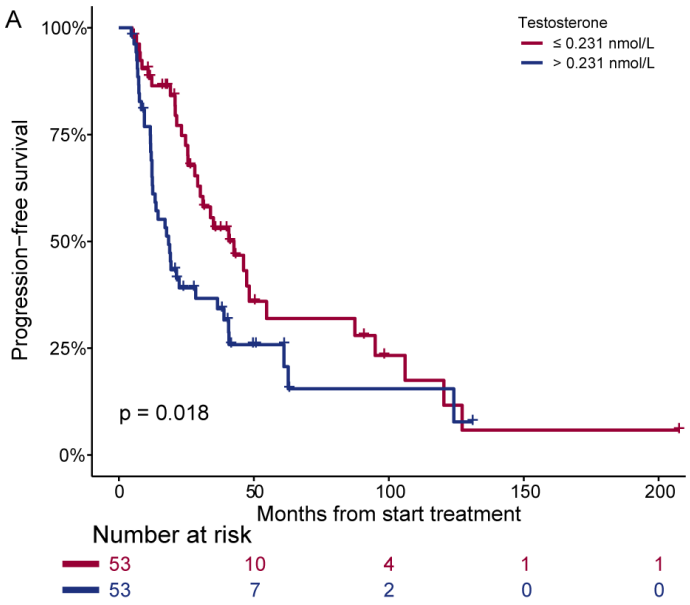
Figure 1. PFS of the testosterone (LC-MS/MS), PSA response and tumor volume risk factors.

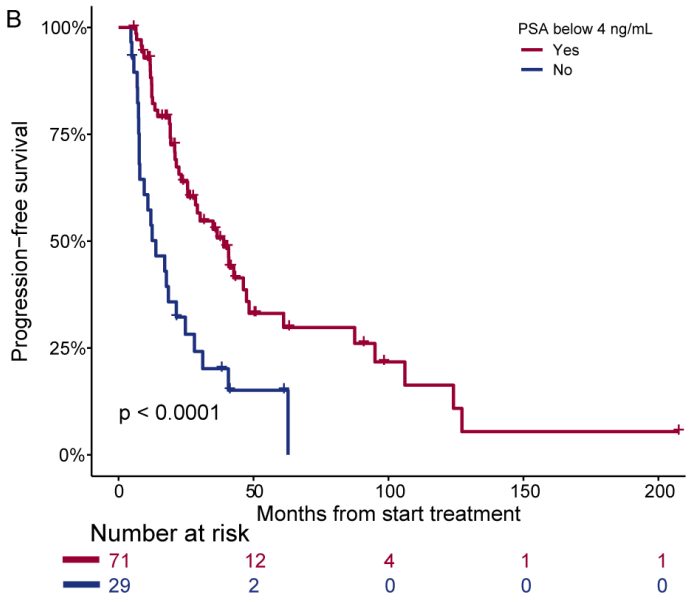
PFS stratified for low (Red, equal to or below median) and high (Blue, above the median) testosterone levels (A). PFS stratified for nadir PSA below 4 ng/mL (Red) and nadir PSA above 4 ng/mL (Blue) (B). PFS stratified for low (Red) and high (Blue) tumor volume (C).

Abbreviations:

PFS = Progression-free survival

PSA = Prostate-specific antigen


A
Testosterone
≤ 0.231 nmol/L
> 0.231 nmol/L
Progression−free survival
100%
75%
50%
25%
0%
p = 0.018
0
50
100
150
200
Months from start treatment
Number at risk
53 10 4 1 1
53 7 2 0 0


B
PSA below 4 ng/mL
Yes
No
Progression−free survival
100%
75%
50%
25%
0%
p < 0.0001
0
50
100
150
200
Months from start treatment
Number at risk
71 12 4 1 1
29 2 0 0 0

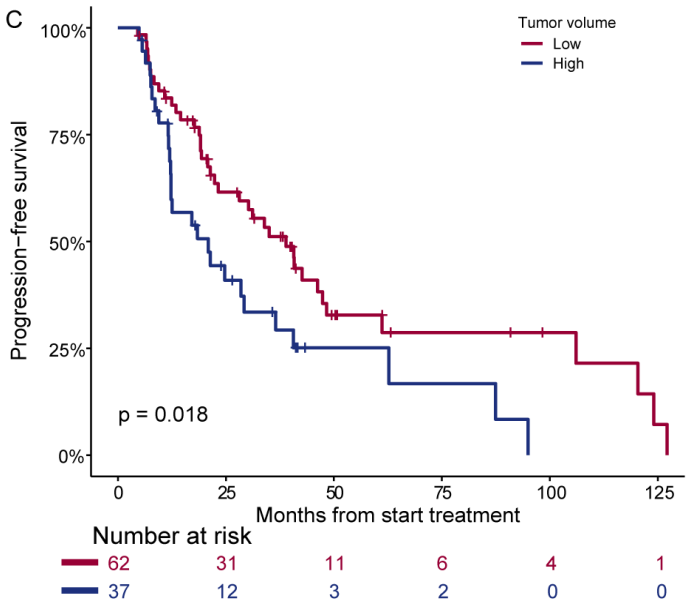
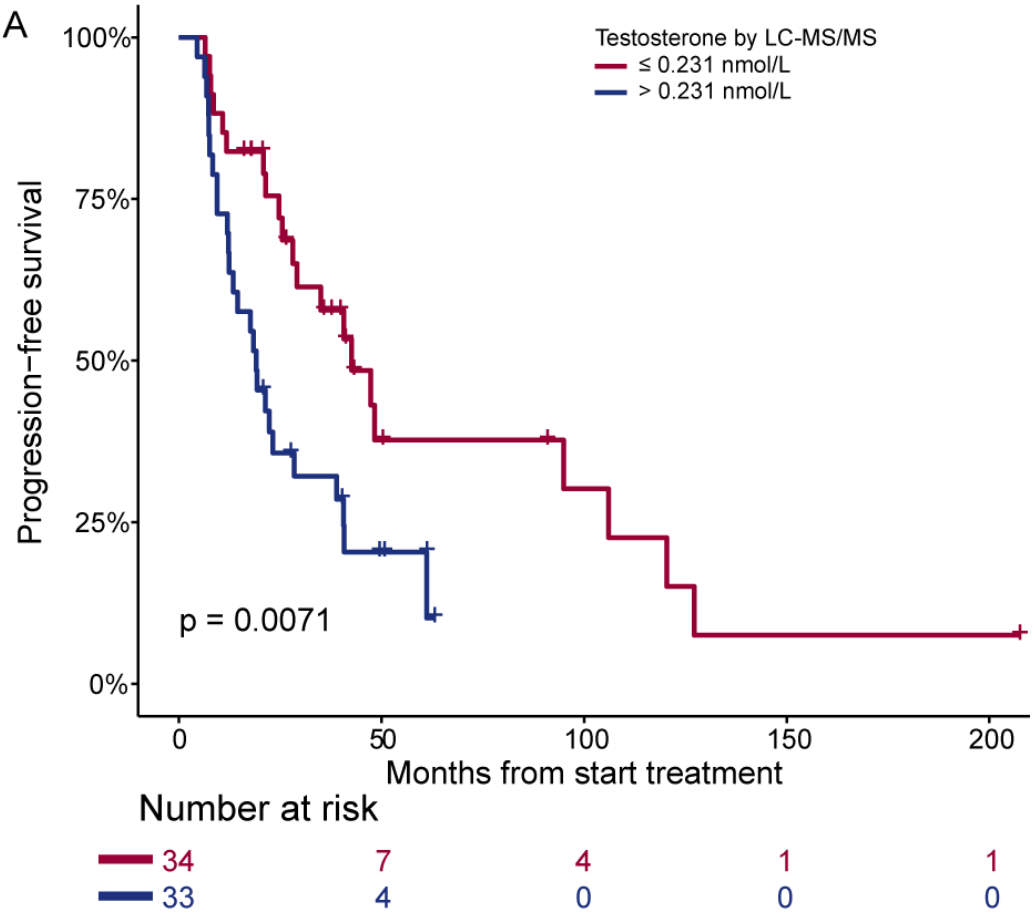

C
Tumor volume
Low
High
Progression−free survival
100%
75%
50%
25%
0%
p = 0.018
0
25
50
75
100
125
Months from start treatment
Number at risk
62 31 11 6 4 1
37 12 3 2 0 0

Figure 2. PFS for testostene by IA and LC-MS/MS.

PFS stratified for low (Red, equal to or below median) and high (Blue, above the median) testosterone levels measured by LC-MS/MS (A). PFS stratified for low (Red, below the assay LLOQ) and high (Blue, above the assay LLOQ) testosterone levels measured by the Roche Cobas IA (B).

Abbreviations:

PFS = Progression-free survival


A
Testosterone by LC-MS/MS
≤ 0.231 nmol/L
> 0.231 nmol/L
Progression−free survival
100%
75%
50%
25%
0%
p = 0.0071
0
50
100
150
200
Months from start treatment
Number at risk
34 7 4 1 1
33 4 0 0 0

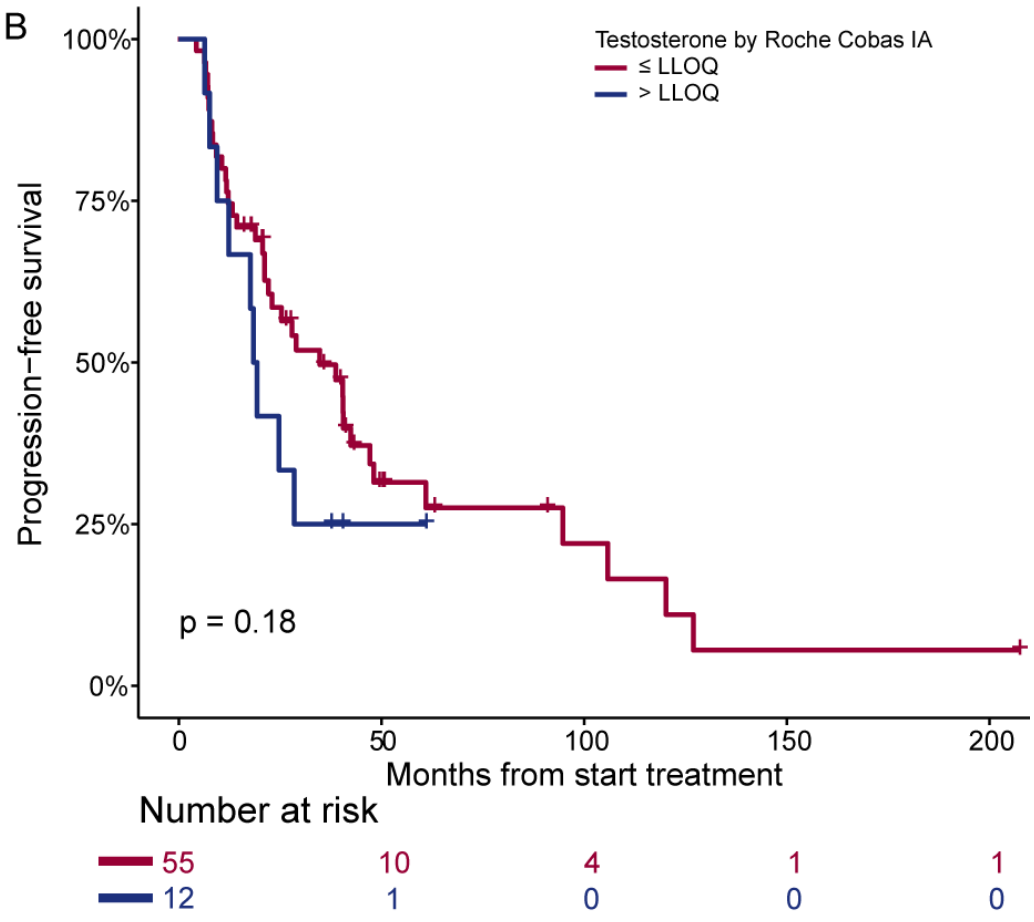

B
Testosterone by Roche Cobas IA
≤ LLOQ
> LLOQ
Progression−free survival
100%
75%
50%
25%
0%
p = 0.18
0
50
100
150
200
Months from start treatment
Number at risk
55 10 4 1 1
12 1 0 0 0

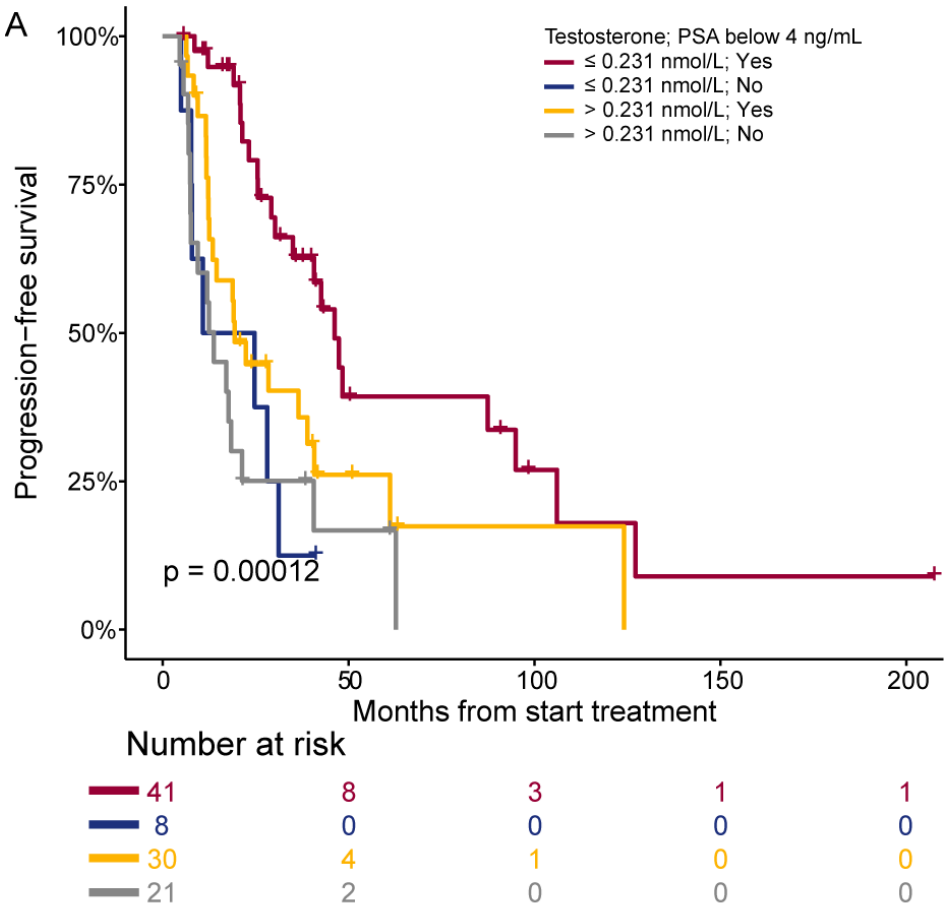
Figure 3. PFS for combinations of the testosterone (LC-MS/MS), PSA response and tumor volume risk factors.

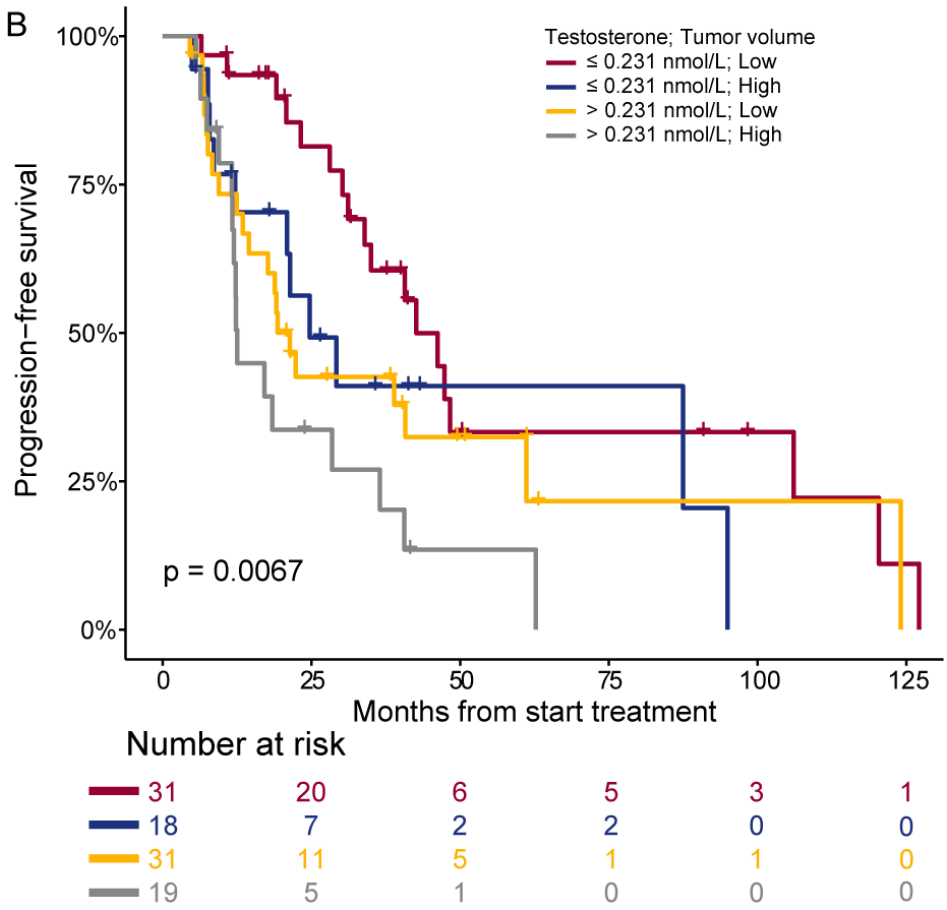
PFS stratified for low and high testosterone levels (cut-off at median) combined with PSA response below 4 ng/mL; low testosterone and PSA below 4 ng/mL (Red), low testosterone and PSA above 4 ng/mL (Blue), high testosterone and PSA below 4 ng/mL (Yellow), and high testosterone and PSA above 4 ng/mL (Gray) (A). PFS stratified for low and high testosterone levels combined with low and high tumor volume; low testosterone and low tumor volume (Red), low testosterone and high tumor volume (Blue), high testosterone and low tumor volume (Yellow), and high testosterone and high tumor volume (Gray) (B).

Abbreviations:

PFS = Progression-free survival

PSA = Prostate-specific antigen

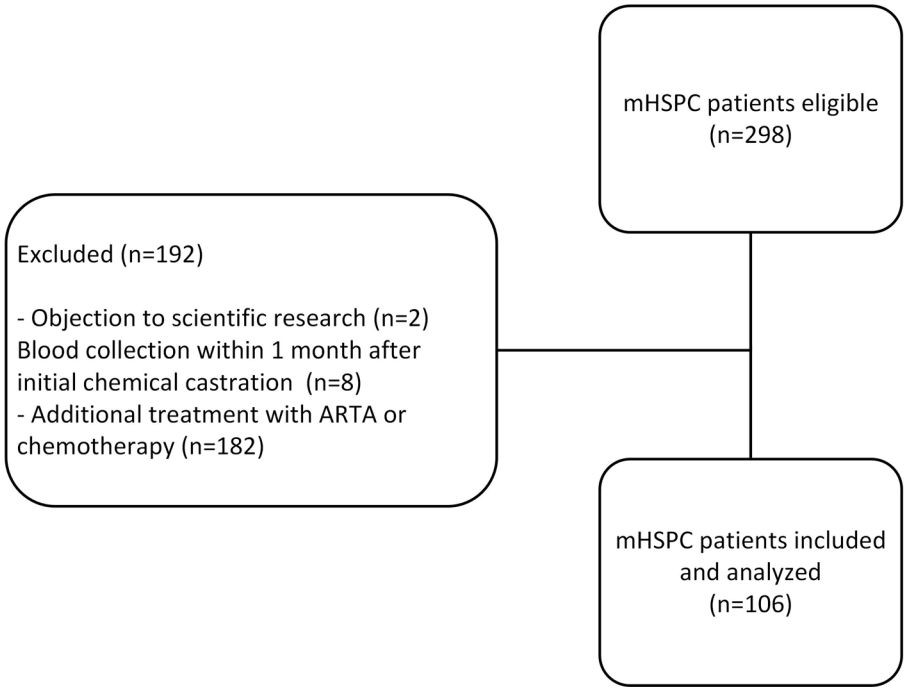

A
Testosterone; PSA below 4 ng/mL
≤ 0.231 nmol/L; Yes
≤ 0.231 nmol/L; No
> 0.231 nmol/L; Yes
> 0.231 nmol/L; No
Progression−free survival
100%
75%
50%
25%
0%
p = 0.00012
0
50
100
150
200
Months from start treatment
Number at risk
41 8 3 1 1
8 0 0 0 0
30 4 1 0 0
21 2 0 0 0


B
Testosterone; Tumor volume
≤ 0.231 nmol/L; Low
≤ 0.231 nmol/L; High
> 0.231 nmol/L; Low
> 0.231 nmol/L; High
Progression−free survival
100%
75%
50%
25%
0%
p = 0.0067
0
25
50
75
100
125
Months from start treatment
Number at risk
31 20 6 5 3 1
18 7 2 2 0 0
31 11 5 1 1 0
19 5 1 0 0 0

Supplementary Material

Supplementary Figure 1. Flow chart displaying participant inclusion and exclusion.

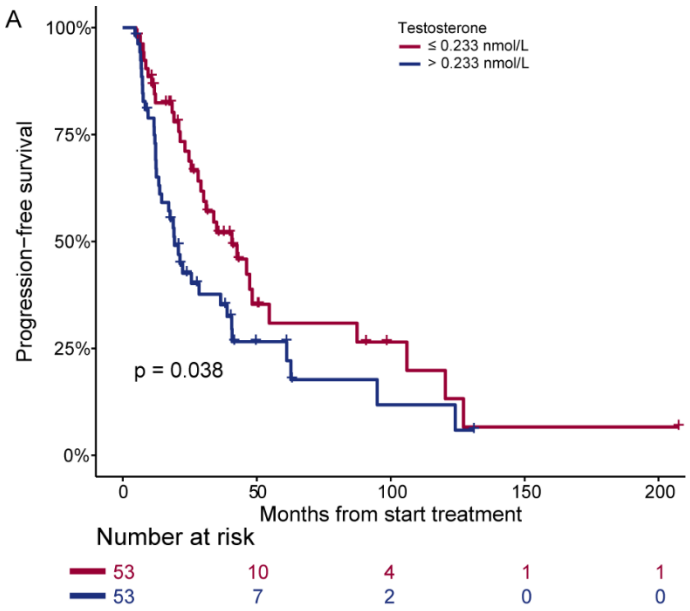
ARTA = Androgen receptor targeting agent

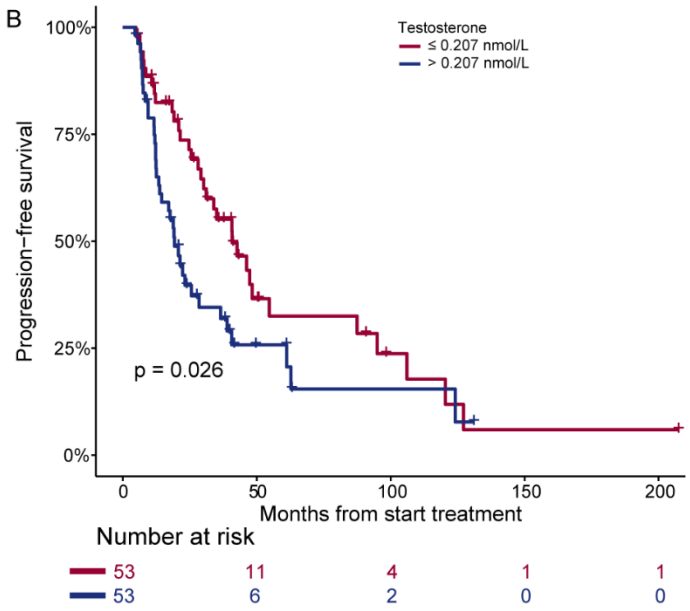


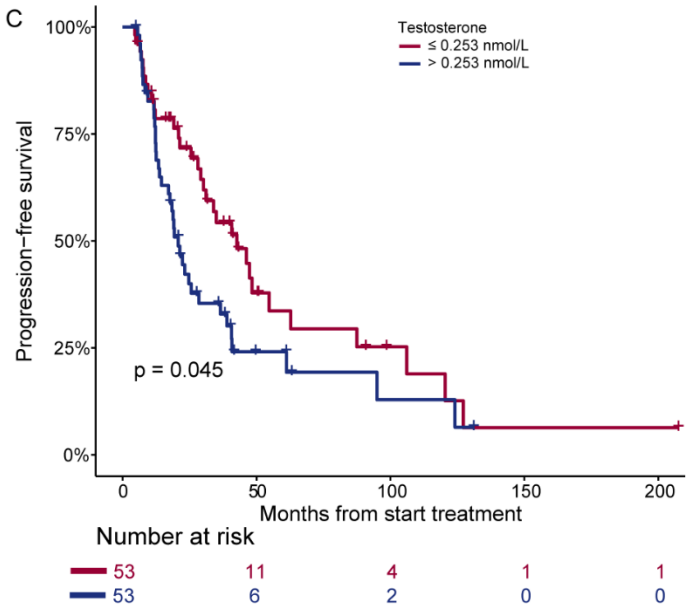
Supplementary Figure 2. PFS stratified for low (Red, equal to or below median) and high (Blue, above the median) median within-patient testosterone levels (A). PFS stratified for low (Red, equal to or below median) and high (Blue, above the median) minimum within-patient testosterone levels (B). PFS stratified for low (Red, equal to or below median) and high (Blue, above the median) maximum within-patient testosterone levels (C).

Abbreviations:

PFS = Progression-free survival


A
Testosterone
≤ 0.233 nmol/L
> 0.233 nmol/L
Progression−free survival
100%
75%
50%
25%
0%
p = 0.038
0
50
100
150
200
Months from start treatment
Number at risk
53 10 4 1 1
53 7 2 0 0


B
Testosterone
≤ 0.207 nmol/L
> 0.207 nmol/L
Progression−free survival
100%
75%
50%
25%
0%
p = 0.026
0
50
100
150
200
Months from start treatment
Number at risk
53 11 4 1 1
53 6 2 0 0


C
Testosterone
≤ 0.253 nmol/L
> 0.253 nmol/L
Progression−free survival
100%
75%
50%
25%
0%
p = 0.045
0
50
100
150
200
Months from start treatment
Number at risk
53 11 4 1 1
53 6 2 0 0

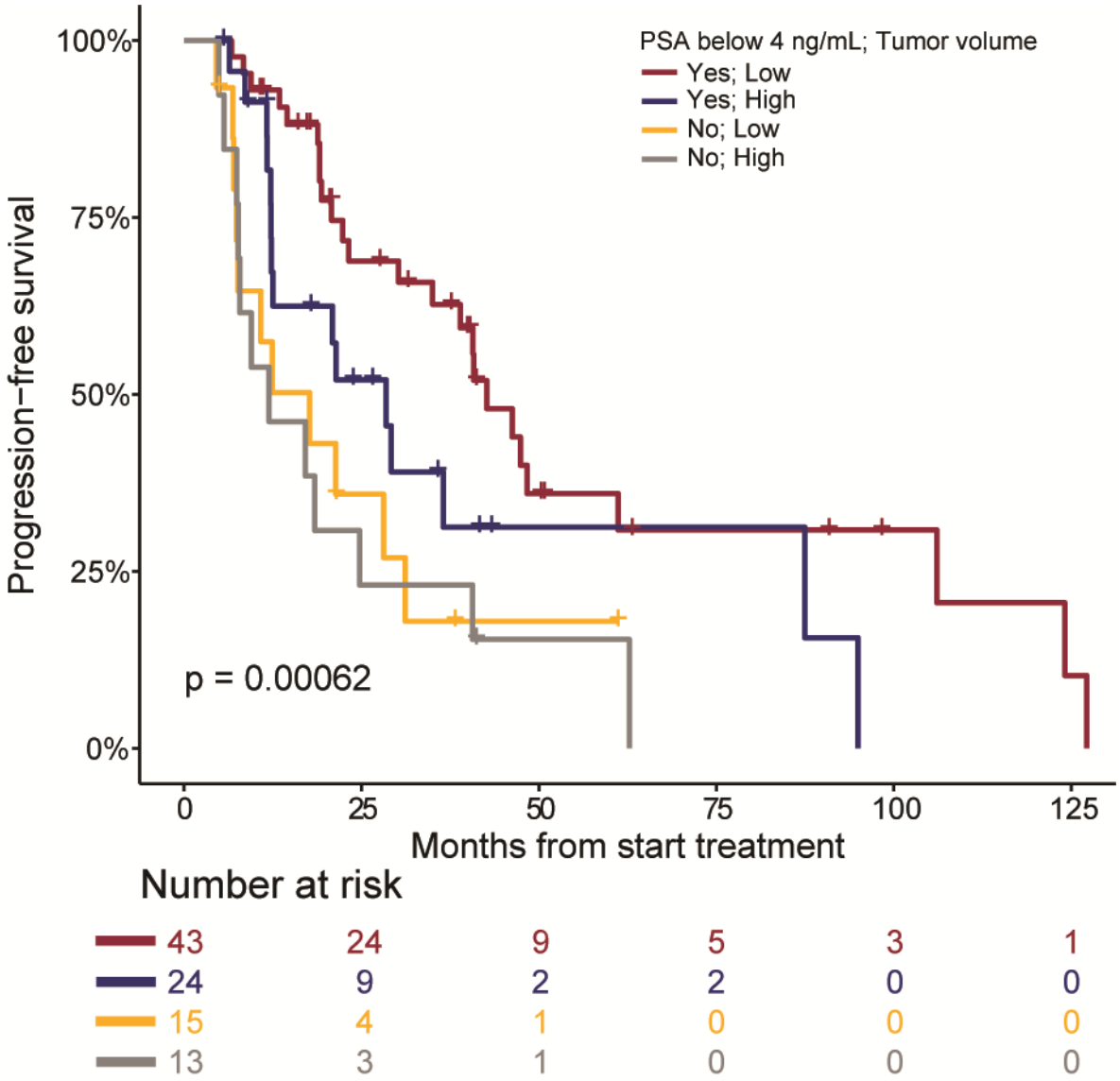
Supplementary Figure 3. PFS stratified for PSA response below 4 ng/LmL combined with low and high tumor volume; PSA below 4 ng/mL and low tumor volume (Red), PSA below 4 ng/mL and high tumor volume (Blue), PSA above 4 ng/mL and low tumor volume (Yellow), and PSA above 4 ng/mL and high tumor volume (Gray).

Abbreviations:

PFS = Progression-free survival

FFS = Failure-free survival

PSA = Prostate-specific antigen



Chapter 2.4

Predictive value of low testosterone concentrations during and prior to enzalutamide treatment in metastatic castration-resistant prostate cancer

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Abbreviations

ADT	androgen deprivation therapy
ALP	alkaline phosphatase
CI	confidence interval
mCRPC	castration-resistant prostate cancer
FFS	failure-free survival
HR	hazard ratio
LC-MS/MS	liquid chromatography tandem–mass spectrometry
LHRH	luteinizing hormone releasing hormone
FFS	failure-free survival
PFS	progression-free survival
PSA	prostate-specific antigen

Abstract

Background: Enzalutamide is an effective treatment for metastatic castration resistant prostate cancer (mCRPC) patients. However, variances in responses are observed and there is a need for biomarkers predicting treatment outcome and selection. In this study, we aimed to explore the predictive value of testosterone for first-line enzalutamide treatment of mCRPC.

Methods: A retrospective analysis of 72 mCRPC patients with no prior abiraterone or docetaxel treatment was performed. Serum testosterone was measured using a liquid chromatography tandem-mass spectrometry method. Association of pre- and during-enzalutimide treatment testosterone levels with progression-free survival (PFS) and failure-free survival (FFS) was investigated using univariate and multivariate Cox models. Testosterone levels were dichotomized into a low (Q1) and high (interquartile range-Q4) group.

Results: Median PFS (7.4 v. 20.8 months, $p<0.0001$) and FFS (6.6 v. 17.7 months, $p<0.0001$) was shorter for patients with low testosterone levels (<0.217 nmol/L) during enzalutamide treatment. Furthermore, univariate Cox proportional hazards models revealed that low testosterone levels were associated with shorter PFS (HR 3.5, 95%CI 1.9-6.3; $p<0.001$) and FFS (HR 3.1, 95%CI 1.7–5.5; $p<0.001$). Pre-treatment testosterone levels were lower than during-treatment levels ($p<0.0001$) and low pre-treatment testosterone levels (<0.143 nmol/L) were associated with shorter median PFS (12.6 v. 20.5 months, $p<0.01$) and FFS (12.6 v. 22.5 months, $p<0.01$).

Conclusion: The results of this study suggest that low serum testosterone levels during and prior to enzalutamide treatment can predict progression in mCRPC patients and identifies tumors resistant to next-in-line enzalutamide treatment. Validation in a prospective cohort is warranted.

Key words: serum testosterone, enzalutamide, castration resistant prostate cancer, LC-MS/MS, survival

Introduction

Since Charles Huggins discovered that prostate cancer growth is driven by androgens, suppression of androgenic activity is the mainstay of prostate cancer patients systemic treatment (1). For hormone-sensitive prostate cancer (HSPC), gonadal androgen deprivation is established by either surgical or chemical castration with luteinizing hormone releasing hormone (LHRH) agonists or antagonists (2, 3). While LHRH analogs provide substantial clinical benefit, patients inevitably progress to metastatic castration resistant prostate cancer (mCRPC) (4). In the last two decades, novel therapeutics targeting residual androgenic activity were developed and approved for clinical practice, including enzalutamide. Enzalutamide is a potent non-steroidal antiandrogen blocking Androgen Receptor (AR) signaling, which showed an overall survival benefit in mCRPC patients (5-7). Although the majority of mCRPC patients benefit from enzalutamide, there is a large variation in response duration. To this end, more recent research is focused on finding and validating biomarkers that can predict treatment outcome and guide treatment selection decisions (8, 9).

One of the biomarkers investigated is serum testosterone. In two previous studies using appropriate testosterone assay techniques, it was found that low tesosterone levels were associated with shorter time to death (10, 11). Although other studies also found an assocation between testosterone levels and survival in enzalutamide treated patients (12, 13), adequate interpretation of results is hampered by their study design and techincal limitations of the applied immunoassays (14, 15). Notably, state-of-the-art technologies, based on liquid chromatography tandem mass spectrometry (LC-MS/MS), enable a more sensitive and accurate analysis of ultra low circulating testosterone allowing accurate quantitation in all castrated prostate cancer patients (15). In this study, we aim to investigate the association of during- and pre-enzalutamide treatment testosterone levels with progression-free and failure-free survival using a highly sensitve and accurate LC-MS/MS assay.

Patients and Methods

Patients

mCRPC patients that visited the Antoni van Leeuwenhoek hospital between March 2018 and April 2020 for which a sensitive testosterone analysis by means of LC-MS/MS was requested by physicians specialised in the treatment of mCRPC, were included. Additonal exclusion criteria were; 1) patient objection to scientific research; 2) no prostate cancer diagnosis; 3) blood collection after 14 months from treatment initation; 4) no treatment with enzalutamide; 5) prior treatment with abiraterone or docetaxel. mCRPC was diagnosed in

patients that exhibited prostate-specific antifgen (PSA) progression while testosterone levels remained below 1.7 nmol/L (4). The study was approved by the institutional review board (IRBd18145) and was in accordance with the declaration of Helsinki.

Testosterone analysis

Samples were stored up to one week at 4 °C or up to one year at – 20 °C. All samples were measured using an LC-MS/MS method previously described (16, 17). In short, the method comprises of liquid-liquid extraction, reversed phase chromatography and consecutive multiple reaction monitoring. The lower limit of quantitation was determined at 0.025 nmol/L.

Data analysis and statistics

Follow-up started at initiation of enzalutamide treatment. In case no event had occurred until the last contact with the patient or a patient was lost to follow-up (eg. Primary treatment elsewhere), patients were censored. Progression was defined according to the published methods from the Prostate Cancer Working Group 3, i.e. a PSA increase of ≥ 25% and at least 2 µg/L above the nadir with a confirmed consecutive rise in PSA, or radiographic progression of soft-tissue lesions and/or detection of at least two lesions on a bone scan (18). Treatment failure was defined as the first of the following events: PSA progression, radiographic progression, non-progression end of treatment and death. For overall survival (OS) analysis, the death of a patient was used to mark events. Progression-free survival (PFS), failure-free survival (FFS) and OS stratified for testosterone levels were evaluated using Kaplan Meijer curves, and corresponding differences were assessed with the log-rank test. Univariate and multivariate Cox proportionate hazards models were established for PFS and FFS to determine the associations of testosterone levels with treatment outcome and to correct for potential confounding risk factors. Risk factors that were incorporated in this study were age (continuous variable; years), Gleason score (categorical variable; 6-7, 8-10 and unknown), prior prostatectomy (Categorical variable; yes or no), time on initial ADT (Continuous variable; days), baseline PSA concentration (Continuous; ng/mL) and baseline alkaline phosphatase (ALP; continuous variable; UI/L). A secondary analysis was initial PSA response. Herein, relative differences between baseline PSA and the first PSA measurement during enzalutamide treatment were calculated. A relative decline of 50% was considered relevant.

Our aim was to include at least 30 patients in this study. This was based on a previous study investigating the association between baseline androgens and survival in 30 abiraterone treated patients (11). Differences between groups were assessed using nonparametric Mann-Whitney U tests and chi-squared tests for continuous and categorical variables,

respectively. Associations were defined as hazards ratios (HR) and corresponding confidence intervals (CI). A p-value below 0.05 was considered statistically significant. All statistical analyses were conducted using R (Version 3.6.3).

Results

In total, 72 patients were included for further analysis and were stratified according to their testosterone levels (Low, ≤ 0.217 nmol/L; high, > 0.217 nmol/L; 6.3 ng/dL), based on the first quartile (Low) and the second to fourth quartile (High). This is a modified version of a previously described approach (10, 11). Median time from the start of enzalutamide treatment to sample collection was 86 (IQR 58 – 190) days. Characterization of these patients is listed in Table 1. Median follow-up was 513 days for both PFS and FFS. Although differences were found for testosterone and ALP, no differences were found for age, type of castration, Gleason score groups, prior prostatectomy, days on prior ADT and PSA levels. All patients had testosterone levels below 1.0 nmol/L.

Relative PSA responses stratified for patients with low and high testosterone levels are displayed in Figure 1. In total, 44% of patients with low during-treatment testosterone levels had a relevant PSA response (< -50%), whereas 72% of patients with high during-treatment testosterone levels showed a relevant PSA response. Kaplan-Meier Curves for PFS and FFS based on low and high testosterone levels are shown in Figure 2. Median time to progression was 7.4 and 20.8 months (low v. high, p<0.0001). Median time to treatment failure was 6.6 and 17.7 months (low v. high, p<0.0001). For median OS, a similar significant difference between testosterone groups was observed (Supplementary Figure 1).

In a subsequent analysis, the Cox proportional hazards model was used to assess the association of low and high testosterone levels with PFS and FFS. Univariate and multivariate analysis of testosterone levels and known risk factors are listed in Table 2. For univariate analysis of PFS, low testosterone was associated with a shorter time to progression (HR 3.5, 95%CI 1.9-6.3; p<0.001) and increased baseline ALP levels were associated with shorter time to progression (HR 1.002, 95%CI 1.001-1.004). Multivariate analysis of testosterone level groups and baseline ALP levels revealed a similar association for testosterone groups (Low, HR 3.7, 95%CI 2.0–6.9; p<0.001) and baseline ALP (HR 1.002, 95%CI 1.001-1.004). For OS and low testosterone levels, similar HR were observed (Supplementary Table 1).

Univariate analysis for FFS showed that low testosterone was associated with shorter time to treatment failure (HR 3.1, 95%CI 1.7–5.5; p<0.001). Furthermore, increases of baseline PSA levels (HR 1.0004, 95%CI 1.0 – 1.001; p<0.01) and ALP (HR 1.002, 95%CI 1.0-1.004)

were associated with shorter time to treatment failure. Notably, multivariate analysis of testosterone groups, baseline PSA and ALP levels showed a similar association between time to treatment failure and low testosterone levels (HR 3.4, 95%CI 1.9-6.3; p<0.001) and baseline PSA (HR 1.0005, 95%CI 1.0002 – 1.008; p<0.001) and ALP (HR 1.002, 95%CI 1.0002-1.004) levels.

For 33 patients, left-over samples and testosterone levels were obtained prior to enzalutamide treatment. Median time from sample collection to the start of enzalutamide treatment was 31 days (IQR 8-57). Pre- and during-treatment differences in testosterone levels and KM curves for PFS and FFS are displayed in Figure 3. Pre-treatment testosterone levels were lower than post-treatment testosterone levels (Figure 3A, p<0.0001). Applying a cut-off at the first quartile (0.143 nmol/L), median time to progression was shorter for low testosterone levels (Figure 3B, low v. high, 12.6 v. 20.5 months, p<0.01). FFS analysis showed similar results (Figure 3C, low v. high, 12.6 v. 22.5 months, p<0.01). Median OS was not different between testosterone groups (Supplementary Figure 2).

Discussion

In this retrospective observational study, we aimed to investigate the predictive value of circulating testosterone levels pre- and during-enzalutamide treatment. For during-treatment analysis, testosterone levels were categorized as low or high according to quartile ranges (Low, minimum to Q1; High, Q1 to maximum). The patients with the lowest circulating testosterone concentrations during treatment (≤ 0.217 nmol/L) were associated with shorter times to progression (HR 3.5, 95%CI 1.9-6.3; p<0.001) and treatment failure (HR 3.1, 95%CI 1.7–5.5; p<0.001). To adjust for potentially confounding risk factors, we established multivariate Cox proportional hazards models. For PFS, baseline ALP levels were added to the model, which resulted in a similar HR (Multivariate HR 3.4 vs. univariate HR 3.5) for low testosterone. Accordingly, multivariate analysis for FFS combining testosterone groups with baseline PSA and ALP levels also resulted in similar HR for low testosterone levels (Multivariate HR 3.2 vs. univariate HR 3.1). In addition, analysis of OS resulted in similar differences in time to death and HR values. This indicates that testosterone levels could be regarded as an independent risk factor and that low levels might be predictive for time to progression and treatment failure for mCRPC patients treated with first-line enzalutamide.

Using samples from these patients that were obtained prior to enzalutamide treatment, we found that low testosterone levels (≤ 0.143 nmol/L) were associated with shorter time to progression compared to testosterone levels higher than 0.143 nmol/L (low v. high, 12.6 v. 20.5 months, p<0.01). Similar results were observed for FFS (low v. high, 12.6 v. 22.5

months, p<0.01). Median OS was not different between testosterone groups. Except for OS, these results are in line with the findings using during-treatment testosterone levels. Notably, pre-treatment levels were lower than during-treatment testosterone levels (Figure 3A, p<0.0001). While increased testosterone levels in prostate cancer patients treated with AR inhibitors have been previously observed, no underlying biological mechanisms have been described (19, 20).

Although data on the prognostic and predictive value of serum testosterone levels during mCRPC treatment is scarce, testosterone has been studied widely as a pharmacodynamic parameter in these patients. This has led to the implementation of serum testosterone as an indicator for adequacy of castration, which is used in combination with PSA or radiographic progression to diagnose mCRPC (4). Interestingly, more recent studies have found indications that baseline serum testosterone was associated with second line hormone therapy outcome in mCRPC patients. For example, Ryan et al. showed with data from the COU-AA-301 trial that patients treated with abiraterone acetate, a CYP17A1 inhibitor suppressing adrenal androgens, with higher baseline testosterone levels demonstrated longer median OS (18.9 vs. 10.4 months for > 0.298 nmol/L and ≤ 0.080 nmol/L, respectively) (10). Other studies attempted similar analyses for patients treated with enzalutamide, abiraterone and docetaxel, but lacked sensitive and accurate testosterone assays and were underpowered (12, 13, 21).

Notably, it has already been hypothesized that high residual testosterone levels under castration treatment originating from the adrenal gland or from within the tumor microenvironment could attribute to increased residual androgenic activity. This testosterone synthesis is independent and not affected by from androgen suppression by LHRH agonists or antagonists (22-24). Therefore, mCRPC patients with a higher remaining circulating testosterone level under castration treatment, might have a better response to enzalutamide treatment than patients with lower circulating testosterone levels due to remaining androgen signaling sensitivity of the tumor. Importantly, previous studies have already generated evidence confirming this hypothesis (12, 13, 21, 25).

It should also be noted that sensitive and accurate testosterone assays are a requirement for conducting studies in castrated prostate cancer patients. While studies comparing commonly applied immunoassays with mass spectrometry-based testosterone assays are lacking for these patients, multiple studies have been performed in women or children demonstrating substantial discrepancies between results (26-28). Furthermore, prominent journals and the Prostate Cancer Working Group recommend mass spectrometry-based steroid analysis when used for primary study endpoints (18). To this end, we compared the results obtained

with our laboratory developed LC-MS/MS assay with a conventional immunoassay, which yielded relative differences up to 111%, demonstrating the limitation of immunoassays for quantifying testosterone castration levels (15).

In addition to the clinical potential of serum testosterone measurement during or prior to enzalutamide treatment, some limitations of this study should be noted. Firstly, the study followed a retrospective data analysis design and patient inclusion was driven by serum testosterone requests made by physicians treating advanced prostate cancer. This could have introduced selection bias as these physicians did not consult all advanced prostate cancer patients in our hospital. Secondly, limited clinical information on Gleason score, metastasis status and prior prostatectomy was available. Therefore, these results should be interpreted with some caution, despite patient characterization showed reasonable homogeneity among testosterone quartile groups. An independent validation study is however necessary to confirm the presented findings. Finally, time of blood collection was not standardized in this study. While this could have led to selection bias, it should be emphasized that testosterone levels remain stable during enzalutamide treatment. In a recent study using at least five longitudinal samples over the whole enzalutamide treatment period, our lab estimated within- and between-patient variation of testosterone levels in these patients (16). Herein, patients demonstrated high individuality of testosterone levels, i.e. within-patient levels constituted only a small part of the population reference interval (29). To this end, samples collected at a later stage during treatment should contain similar testosterone levels as samples collected at the onset of treatment enabling surrogate time of blood collection standardization.

To conclude, we show that mCRPC patients with testosterone concentrations below 0.217 nmol/L during or below 0.143 nmol/L prior to enzalutamide treatment might have a significant lower PFS and FFS and represent a population rather resistant to next-in-line endocrine treatment. There is increasing evidence that testosterone levels under ADT can predict treatment outcome for additional hormone therapies, such as abiraterone (10, 11). In addition to the current evidence, these results add to the suggestion that a more prominent role of testosterone monitoring might be appropriate for mCRPC patients, although prospective studies are needed to validate our findings.

Acknowledgements

None.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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Table 1. Patient characterization.

Abbreviations:

ALP = alkaline posphatase

PSA = prostate-specific antigen.

	Testosterone			
Characteristic	**≤ 0.217 nmol/L**	**> 0.217 nmol/L**	**p-value**	**overall p-value**
No. of patients	18	54		
Age, yr - median (IQR)	69 (65-71)	72 (65-76)	0.10	
Type of castration - n (%) *				
LHRH agonist	16 (89)	49 (91)		1
LHRH antagonist	2 (11)	5 (9)		
Orchiectomy	0 (0)	0 (0)		
Gleason Score - n (%) *				
6-8	10 (56)	26 (48)		0.75
9-10	8 (44)	27 (50)		
Unknown	0 (0)	1 (2)		
Prior prostatectomy - n (%) *				
Yes	6 (33)	18 (35)		1
No	12 (67)	35 (65)		
Days on hormone-sensitive ADT - median (IQR)	520 (358-876)	560 (325-1012)	0.89	
Baseline PSA, ng/mL - median (IQR)	37.1 (16.1-61.3)	26.5 (12.3-61.5)	0.52	
Baseline ALP, U/l - median (IQR)	104 (89-115)	85 (65-103)	<0.05	
Testosterone, nmol/L - median (IQR) **	0.173 (0.149-0.194)	0.399 (0.283-0.509)	<0.001	
SI conversion factors: To convert testosterone to ng/dL, multiply values by 28.8184.				
* Percentage sums may deviate from 100 due to rounding off				
** Testosterone was quantitated using an ultrasensitive LC-MS/MS assay				

Table 2. Cox proportional harzards analysis for progression free survival and failure-free survival.

Abbreviations:

PSA = Prostate-specific antigen

			Progression-free Survival					Failure-free Survival			
			Univariate analysis		Multivariate analysis			Univariate analysis		Multivariate analysis	
Variable	Category	Events	HR (95%CI)	p value	HR (95%CI)	p value	Events	HR (95%CI)	p value	HR (95%CI)	p value
Testosterone (nmol/L)	≤0.217	17/18	3.5 (1.9-6.3)	<0.001	3.4 (1.9-6.3)	<0.001	18/18	3.1 (1.7-5.5)	<0.001	3.2 (1.8-5.8)	<0.001
	>0.217	33/54	1.0 (ref)		1.0 (ref)		39/54	1.0 (ref)		1.0 (ref)	
Age (years)	Continuous	50/72	1.0 (0.98-1.1)	0.46			57/72	1.0 (0.98-1.1)	0.52		
Gleason score	6-7	23/36	0.75 (0.43-1.3)	0.32			37/49	0.87 (0.51-1.5)	0.62		
	8-10	26/35	1.0 (ref)				18/21	1.0 (ref)			
	Unknown	1/1	1.6 (0.21-11)				2/2	1.5 (0.20-11)			
Prior prostatectomy	Yes	17/25	0.97 (0.54-1.8)	0.92			20/25	1.1 (0.61-1.8)	0.83		
	No	33/47	1.0 (ref)				37/47	1.0 (ref)			
Time on first line treatment	Continuous	50/72	1.0 (1.0-1.0)	0.49			57/72	1.0 (1.0-1.0)	0.49		
Baseline PSA (ng/mL)	Continuous	50/72	1.0 (1.0-1.0)	0.65			57/72	1.0 (1.0-1.0)[c]	0.002	1.0 (1.0-1.0)[e]	<0.001
Baseline ALP (IU/L)	Continuous	50/72	1.0 (1.0-1.0)[a]	0.003	1.0 (1.0-1.0)[b]	0.009	57/72	1.0 (1.0-1.0)[d]	0.01	1.0 (1.0-1.0)[f]	<0.01
[a] 1.002 (1.001-1.004)											
[b] 1.003 (1.001-1.004)											
[c] 1.0004 (1.0-1.001)											
[d] 1.002 (1.0-1.004)											
[e] 1.0004 (1.0001-1.001)											
[f] 1.002 (1.001-1.004)											

Figure 1. PSA response according to low (Red, ≤ 0.217 nmol/L) and high (Blue, > 0.217 nmol/L) testosterone levels. All PSA responses lower than -50% (dashed line) were considered relevant. PSA response was defined as the relative difference between the baseline PSA measurement and the first PSA measurement during enzalutamide treatment.

Abbreviations:

PSA = Prostate-specific antigen

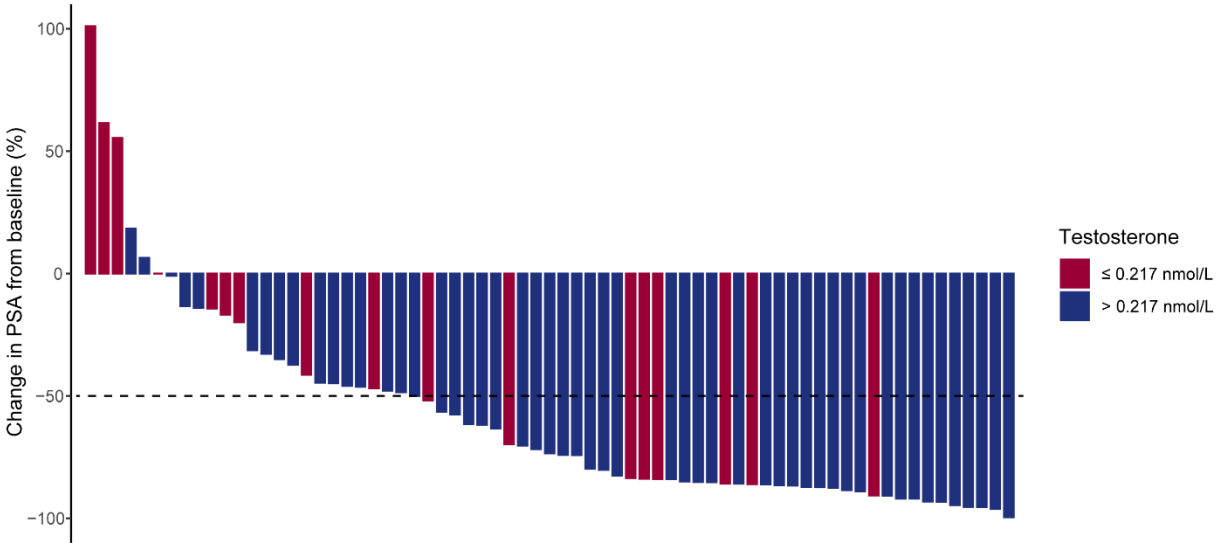
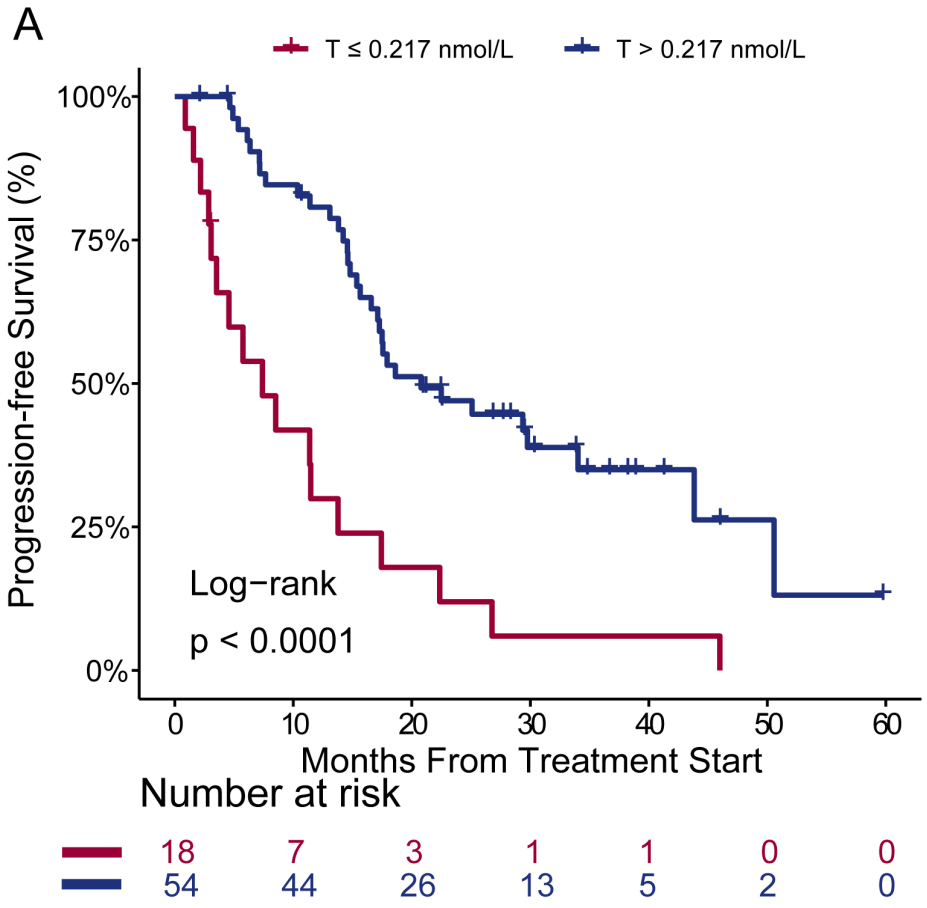


Figure 2. PFS (A) and FFS (B) according to low (Red, minimum to 25th percentile) and high (Blue, 25th percentile to maximum) testosterone levels in the first collected sample during enzalutamide treatment.

Abbreviations:

PFS = Progression-free survival

FFS = Failure-free survival


A
T ≤ 0.217 nmol/L
T > 0.217 nmol/L
Progression-free Survival (%)
100%
75%
50%
25%
0%
Log−rank
p < 0.0001
0
10
20
30
40
50
60
Months From Treatment Start
Number at risk
18 7 3 1 1 0 0
54 44 26 13 5 2 0

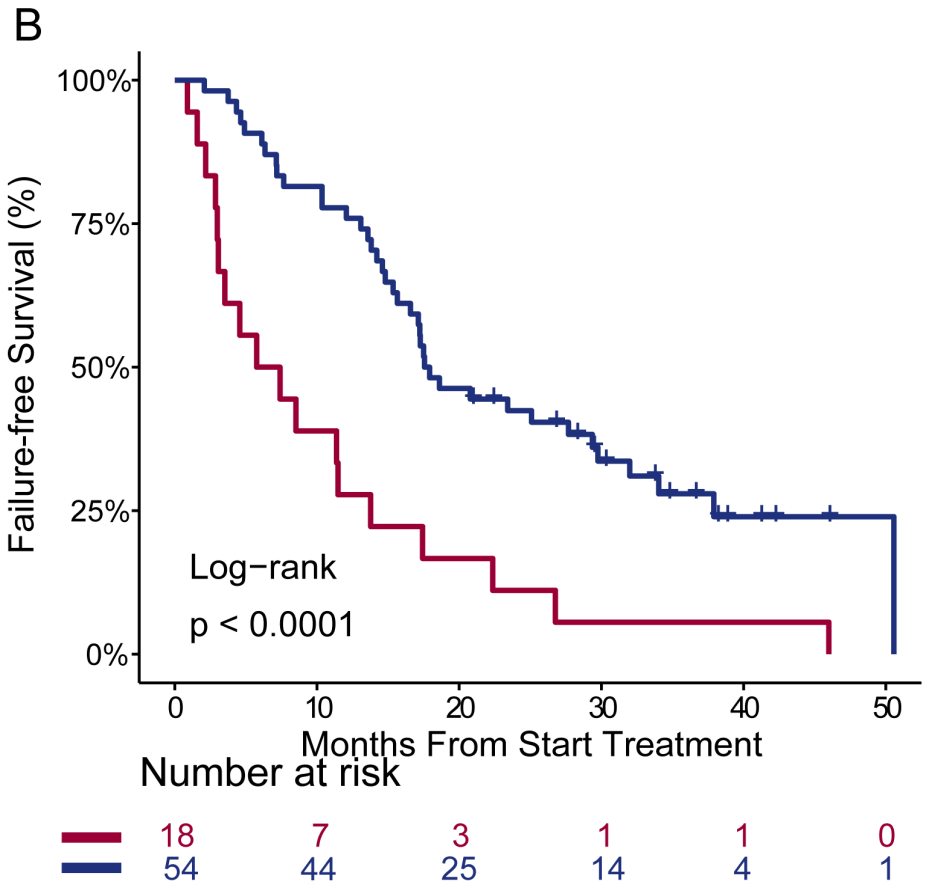

B
Failure-free Survival (%)
100%
75%
50%
25%
0%
Log−rank
p < 0.0001
0
10
20
30
40
50
Months From Start Treatment
Number at risk
18 7 3 1 1 0
54 44 25 14 4 1

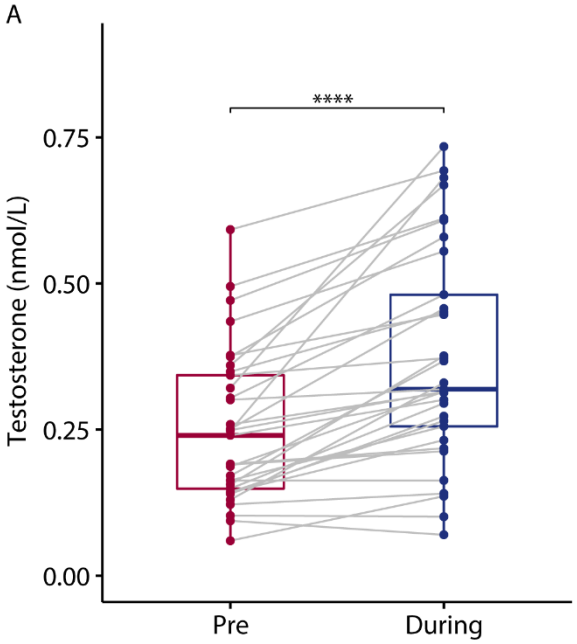
Figure 3. Paired boxplot displaying differences between testosterone levels pre- and post-enzalutamide treatment (A). PFS according to low (Red, ≤0.143 nmol/L) and high (Blue, >0.143 nmol/L) testosterone levels (B). FFS according to low (Red, ≤0.143 nmol/L) and high (Blue, >0.143 nmol/L) testosterone levels (C). Significant pair-wise comparisons are indicated with * symbols (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$).

Abbreviations:

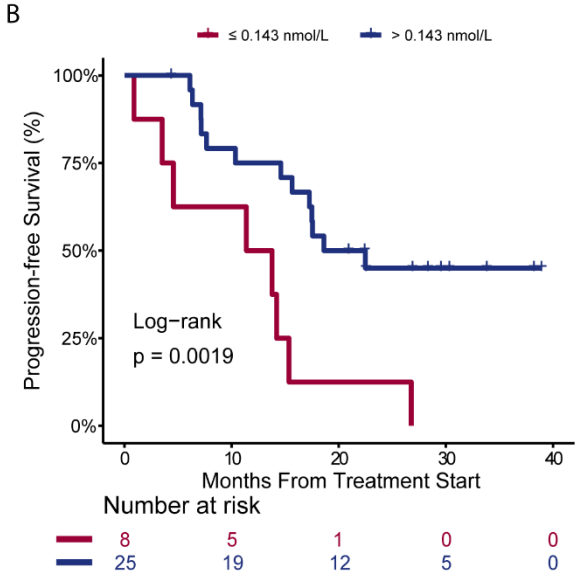
PFS = Progression-free survival

FFS = Failure-free survival

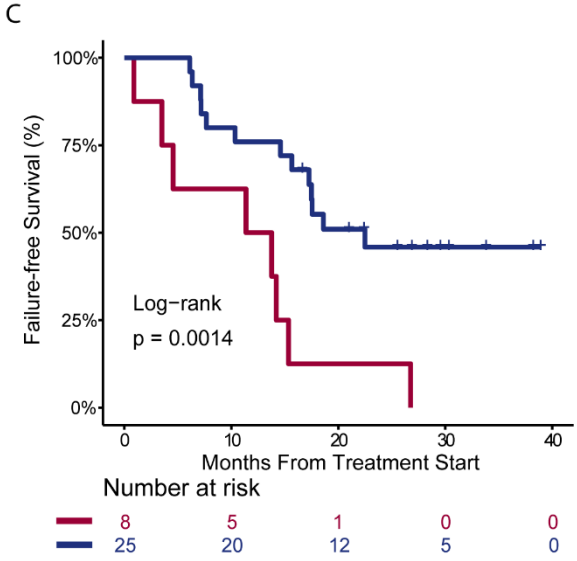
A


****
0.75
0.50
0.25
0.00
Testosterone (nmol/L)
Pre
During

B


≤ 0.143 nmol/L
> 0.143 nmol/L
100%
75%
50%
25%
0%
Progression-free Survival (%)
Log−rank
p = 0.0019
0
10
20
30
40
Months From Treatment Start
Number at risk
8 5 1 0 0
25 19 12 5 0

C


100%
75%
50%
25%
0%
Failure-free Survival (%)
Log−rank
p = 0.0014
0
10
20
30
40
Months From Treatment Start
Number at risk
8 5 1 0 0
25 20 12 5 0

Section 3: Steroid analysis by LC-MS/MS in women at risk of ovarian cancer

Chapter 3.1

Changes in Sex Steroids and relation with Menopausal Complaints in Women Undergoing Risk-Reducing Salpingo-Oophorectomy

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Abstract

Context: Risk-reducing salpingo-oophorectomy (RRSO) is performed in *BRCA1* or *BRCA2* mutant carriers to minimize ovarian cancer risk. Although studies have been performed investigating sex steroid levels, menopausal complaints and sexual functioning in relation to RRSO, their exact relationship remains unknown.

Objectives: To investigate the impact of RRSO on serum sex steroid levels and their association with menopausal complaints and sexual functioning.

Methods: This prospective observational cohort study included 57 pre- and 37 postmenopausal women at risk of ovarian cancer and opting for RRSO. Data collection involved validated questionnaires on sexual functioning and menopausal complaints. Testosterone, androstenedione, estradiol and estrone levels in serum determined by liquid chromatography-tandem mass spectrometry were obtained one day before, six weeks and seven months after RRSO.

Results: In premenopausal women, all four steroids were decreased both six weeks ($p<0.01$) and seven months ($p<0.01$) after RRSO. Furthermore, in these women, decreases in estrogens were associated with a decrease in sexual functioning seven months after RRSO ($p<0.05$). In postmenopausal women, only testosterone was decreased six weeks and seven months ($p<0.05$) after RRSO, which was associated with an increase in menopausal complaints at seven months post-RRSO ($p<0.05$).

Conclusion: Our results suggest that in premenopausal women, decreases in estrogens are related to a decrease in sexual functioning and that in postmenopausal women, testosterone is decreased after RRSO, which indicates that postmenopausal ovaries maintain some testosterone production. Furthermore, in postmenopausal women, a strong decrease of testosterone was associated with more menopausal complaints indicating that future studies investigating testosterone supplementation are warranted.

Keywords: Risk-reducing Salpingo-oophorectomy, menopausal complaints, sexual functioning, androgens, estrogens

Abbreviations

BMI	body mass index
E1	estrone
E2	estradiol
ES	effect sizes
FACT-ES	Functional Assessment of Cancer-Therapy-Endocrine Symptoms
HBOC	hereditary breast and ovarian cancer
HFRS	Hot Flush Rating Scale
HRT	hormone replacement therapy
IA	immunoassay
LC-MS/MS	liquid chromatography tandem-mass spectrometry
OC	ovarian carcinoma
RRSO	risk-reducing salpingo-oophorectomy
SFQ	Sexual Functioning Questionnaire

Introduction

Approximately 10% -15% of all ovarian carcinomas (OC) are due to inherited predisposition (1-3). Ovarian cancer screening has not been proven to be effective in detecting OC at an earlier stage and hereby improving prognosis (4, 5). Therefore, risk-reducing salpingo-oophorectomy (RRSO) is recommended to lower the risk of OC (6, 7). After RRSO, the risk of OC is reduced by 80%-96% (8-10). The recommended age for RRSO after childbearing in BRCA1 carriers is between 35-40 years, and in BRCA2 carriers between 40-45 years. Women from a hereditary breast and ovarian cancer (HBOC) family (two or more first degree relatives with OC) are advised to undergo RRSO after childbearing is completed, but no specific age is given (8, 10).

A major side-effect of RRSO in premenopausal women is the immediate onset of menopause. This is accompanied by an increase in non-cancer related morbidity, including a range of endocrine symptoms, sexual symptoms, mood disturbance, increased risk of cardiovascular disease and osteoporosis (11-13). For example, women generally experience a decline in sexual function after RRSO. Notably, the use of hormone replacement therapy (HRT) mitigates some of these symptoms (14-18). The association of serum sex steroid levels with sexual function has been controversial. Some authors have shown an association between sex steroids and either female sexual dysfunction or hypoactive sexual desire disorder (19, 20). Others did not find an association between sexual domain scores and sex steroid levels (21, 22). A better understanding of factors that influence the severity of symptoms following RRSO could improve patient counselling and possibly treatment of symptoms.

In addition, it has also been debated whether the postmenopausal ovary still produces androgens, especially testosterone. Judd et al. were the first to demonstrate a decline in concentrations of circulating testosterone and androstenedione in postmenopausal women following bilateral oophorectomy (23). Their findings were supported by other reports (24, 25). However, Couzinet et al. presented strong evidence that the postmenopausal ovary does not contribute to circulating androgen levels (26). Notably, most of these studies present an important limitation that scarcely has been addressed. Serum testosterone, and other sex steroids in serum, have been primarily analyzed using immunoassay (IA) technology, which tend to lack sensitivity and specificity in low concentration ranges. This limitation has been extensively described in literature and emphasizes substantial discrepancies when serum testosterone is measured in women (27-31). To increase reliability of these measurements, there has recently been a shift towards routine application

of liquid chromatography tandem-mass spectrometry (LC-MS/MS)-based analysis of steroid hormones, including testosterone (32-34). This technique is currently considered to be the best practice for steroid analysis and is recommended for pediatric and female testosterone analysis (32).

In the present study, our objective was to investigate longitudinal sex steroid levels in premenopausal and postmenopausal women undergoing RRSO using highly sensitive and specific LC-MS/MS methods. In addition, we investigated differences in steroid levels between naturally postmenopausal women (postmenopausal group before RRSO) and women in which menopause was surgically induced by RRSO (premenopausal group). Finally, we aimed to examine the relationship between changes in these sex steroid levels and both sexual functioning and menopausal symptoms validated questionnaire scores.

Methods

Research setting and study sample

This prospective, observational, multicenter study was performed at The Netherlands Cancer Institute (Amsterdam) and the Leiden University Medical Center in the Netherlands. Participants were included between November 2006 and April 2012 (35). Pre- and postmenopausal patients undergoing RRSO with a *BRCA1* or 2 mutation, or women with a familial risk which was estimated to exceed 10% were eligible (36). Women visiting the gynecology outpatient clinic opting for RRSO were invited to participate in the study. All participants provided written informed consent.

Inclusion criteria for the current study were 1) the absence of malignancies at the time of RRSO and 2) no HRT use or hormonal therapy during the study period. Postmenopausal status was defined by amenorrhea for at least twelve months, and pre- and postmenopausal groups were defined based on menopausal status at baseline. Blood samples and questionnaire scores were obtained within one week before (T0), six weeks after (T1) and seven months after (T2) RRSO. The study was in accordance with the declaration of Helsinki and the institutional review boards of the Leiden University Medical Center and the Netherlands Cancer Institute approved the study. Written consent was obtained from each participant.

Measures

The respondents' age, education, relationship status, parity, body mass index (BMI), comorbidities, mutation status (*BRCA1/2*), regular menses, history of breast cancer, previous

breast cancer treatments and current menopausal status were obtained by self-report. Women were asked if they had regular menses during the past three months. If a negative response was received, inquiries were made about the reason of the absent menses.

Between 9:00 am and 5:00 pm, blood was collected in serum separator tubes, centrifuged (10 minutes at 2,500 g) and stored at – 30 °C until analysis. Serum testosterone, estradiol (E2) and estrone (E1) analysis was performed using previously published methods (37, 38). A full description of the method and validation for the quantitation of androstenedione in serum is described in the Supplementary Material (Supplementary Data 1)(39). All steroids were measured using a LC-MS/MS assay. The lower limit of quantitation for each steroid was determined at 0.025 nmol/L (Testosterone), 0.35 nmol/L (Androstenedione), 8.0 pmol/L (E2) and 6.9 pmol/L (E1).

The perceived intensity of hot flushes was assessed using the Hot Flush Rating Scale (HFRS) (40). This scale is used to generate a mean of three scores (1-10 scale) to rate to what extent hot flushes were bothersome and caused interference with daily life in the preceding week. Lower scores indicated less intense symptoms, whereas higher scores indicated more intense symptoms.

We assessed sexual functioning with the sexual functioning questionnaire (SFQ) (41). The SFQ consists of 7 domains: desire (SFQ Desire, 6-items); arousal-sensation (SFQ Arousal S, 4 items); arousal-lubrication (SFQ Arousal L, 2 items); orgasm (SFQ Orgasm, 3 items); enjoyment (SFQ Enjoyment, 6 items); pain (SFQ Pain, 3 items); and partner relationship (SFQ Partner, 2 items). Higher scores indicate better sexual functioning (41).

The Functional Assessment of Cancer-Therapy-Endocrine Symptoms (FACT-ES) was used to monitor menopausal symptoms, the questionnaire consists of 18 items that address a range of menopausal symptoms. Occurrence of each symptom in the past four weeks was scored on a 5-point scale, ranging from ‘not at all’ to ‘very much’. Item scores were summed to obtain a total score (range: 0 – 72), with higher values indicating more menopausal symptoms (42).

Statistics

Pre- and postmenopausal groups were analysed independently. For description of baseline characteristics, data of continuous variables were checked for normality using Q-Q plots and Shapiro-Wilk tests. Normally distributed data were described by mean and standard deviation (SD). In contrast, data that were not normally distributed were described by median and interquartile range (IQR). For longitudinal analysis of steroids, differences between time

points were checked for normality. In case normality could be assumed, data were investigated using repeated measures ANOVA models to assess within-group (Follow-up) effects. Generalized eta squared (η_g^2) values were calculated to describe the effect size (ES) of the ANOVA output. An ES ≤0.08 was considered low, 0.09-0.16 medium and ≥0.17 high, respectively. Post hoc pair-wise paired t-tests (Bonferroni correction) were performed between follow-up moments to evaluate short-term (∆T0-1), long-term (∆T0-2) and follow-up (∆T1-2) differences between steroid levels. In case steroid differences between time points were not normally distributed, only nonparametric pair-wise sign tests were performed. Differences in steroid levels between naturally postmenopausal women (Postmenopause T0) and surgically postmenopausal women (Premenopause T1 and T2) were assessed using unpaired t-tests or Mann Whitney U tests depending on whether normality could be assumed. Differences in questionnaire scores between follow-up moments were evaluated using paired t-tests and Wilcoxon Sign Rank tests depending on the normality of the differences between the repeated measures. Spearman correlation coefficients (ρ) and its corresponding difference with 0 ($p<0.05$ was significant) were used to assess associations between changes in steroid levels and questionnaires in two follow up moments (∆T0-1, ∆T0-2 and ∆T1-2). Correlation coefficients were color coded at a three point level; blue indicating $\rho<0$, white indicating $\rho\approx0$ and red indicating $\rho>0$. Significant correlations were marked using asterisk symbols (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$). To adjust for multiple hypothesis testing, heat map interpretation was focused at correlation of either both androgens or estrogens with a questionnaire score between two follow-up moments. All statistical analyses were performed using R (Version 4.1.0). Spearman's Rho correlation coefficients were color-coded in MS Excel (Version 2016) to generate heat maps.

Results

Between November 2006 and April 2012, 142 of the 210 eligible women were enrolled onto the study, of whom 92 and 50 were pre- and postmenopausal at RRSO, respectively (Fig. 1). Of these women, 48 were excluded based on predefined exclusion criteria, predominantly incomplete follow-up (n=28; Lack of interest or unknown reasons) or HRT use during the trial (n=18). In total, 94 (57 premenopausal; 37 postmenopausal) were included for final data analysis. Baseline characteristics separated for pre- and postmenopausal women are listed in Table 1. Age, DNA status and comorbidities were significantly different between menopausal groups.

In Fig. 2, box plots for each steroid at baseline (T0), six weeks (T1) and seven months (T2) after RRSO are displayed. For testosterone, ANOVA analysis showed that serum

concentrations were affected by follow-up (Fig. 2A, p<0.0001, medium ES, η_g^2=0.1). Specifically, a decrease in serum testosterone levels was observed both after six weeks (Premenopause, p<0.01; Postmenopause, p<0.001) and seven months (Premenopause, p<0.001; Postmenopause, p<0.01) after RRSO. ANOVA analysis showed a similar association of follow-up with serum androstenedione levels (Fig. 2B, p<0.0001, low ES, η_g^2=0.06). Herein, only in premenopausal women a decrease in serum androstenedione levels six weeks (p<0.001) and seven months after RRSO (p<0.01) was observed. For both E1 and E2 (Fig. 2C and 2D), serum concentrations deviated from a normal distribution, and thus no ANOVA analyses were performed. Pairwise comparisons between follow-up moments revealed decreases six weeks (p<0.0001) and seven months (p<0.0001) after RRSO for both estrogens in premenopausal women. No differences in serum estrogen levels between follow-up moments were observed in postmenopausal women.

Relative individual changes between short- or long term follow-up after RRSO and baseline serum sex steroid levels are displayed in Fig. 3. While most premenopausal women demonstrated relative declines in serum steroid levels (Fig. 3A, 81-93%), a small group (Fig. 3A, 7-19%) had no change or an increase in serum steroid level. In 30-46% of the postmenopausal women (Fig. 3B), serum androstenedione, E1 and E2 was similar or increased both short- and long term. In contrast, 16% and 27% of postmenopausal women (Fig. 3B) had increases in serum testosterone levels six weeks and seven months after RRSO, respectively.

Fig. 4 shows boxplots and differences in serum sex steroid levels between postmenopausal women at T0 (naturally postmenopausal) and premenopausal women at T1 and T2 (surgically postmenopausal). While serum testosterone and androstenedione levels were not different between these groups (Fig. 4A and 4B), E2 levels were lower in premenopausal T1 (p<0.01) and T2 (p<0.05) compared to postmenopausal T0 (Fig. 4C). Furthermore, E1 levels were lower in premenopausal T1 (p<0.05), although not in premenopausal T2 (Fig. 4D) compared to postmenopausal T0.

Box plots of questionnaire scores are displayed in Fig. 5 and Supplementary Fig.1 (39). Herein, all sexual functioning questionnaire and menopausal complaint scores decreased, while Hot Flush Rating Scale (HFRS) sum scores increased in premenopausal women. In contrast, no differences were observed in postmenopausal women.

Finally, Fig. 6 lists correlations of follow-up moment differences (∆T0-1, ∆T0-2 and ∆T1-2) between steroid levels and questionnaire scores. In postmenopausal women, long term (∆T0-2) changes in HFRSsum scores and serum androgens were negatively correlated

(p<0.05). In addition, long term (ΔT0-2) SFQ scores and serum estrogen levels were positively correlated (SFQ Desire, p<0.05; SFQ Arousal L, p<0.01; SFQ Arousal S, p<0.01) in premenopausal women. In contrast, short term (ΔT0-1) SFQ scores were positively correlated with serum androgen levels (SFQ Arousal L, p<0.05) and serum estrogen levels (SFQ orgasm, p<0.05) in postmenopausal women. Correlations between steroids and SFQ pain, partner and enjoyment scores were separately analyzed and are shown in Supplementary Fig. 2 (39). In premenopausal women, both estrogens were associated with SFQ Pain (ΔT0-2, p<0.05) and SFQ Enjoyment (ΔT0-1, p<0.05; ΔT0-2, p<0.05).

Discussion

The first aim of this study was to investigate the longitudinal changes in steroid levels in pre- and postmenopausal women at increased risk of hereditary or familiar risk of ovarian cancer who underwent RRSO. In premenopausal women, decreases were observed for all serum sex steroids, both (six weeks after RRSO) and long term (seven months after RRSO), while in postmenopausal women only serum testosterone levels were decreased after RRSO compared to baseline before RRSO. The results in postmenopausal women for testosterone, androstenedione and E2 were in line with the findings of Judd et al. and Fogle et al., whereas Stanczyk et al. observed similar findings for testosterone, androstenedione and E1 (23, 25, 43). Notably, the study of Stanczyk et al. also investigated circulating sex steroid levels pre- and post RRSO in premenopausal women and expectedly found decreased levels for all four sex steroids. Furthermore, Davison et al. and Laughlin et al. also found lower circulating testosterone levels in older women (>55 years old) after RRSO, although androstenedione was not decreased (24, 44). The latter authors also investigated E1 and E2 and found no differences between postmenopausal women with and without ovaries. Interestingly, Couzinet et al. found no difference for both testosterone and androstenedione between postmenopausal women and oophorectomized women (26). Discrepancies of our findings with previous results could be explained by the analytical methodology used. All previous studies depend on radioimmunoassays (RIA) for the quantitation of circulating sex steroids. While RIA, or immunoassays in general, provide considerable sensitivity, they lack specificity in low concentration ranges leading to unreliable results. To this end, mass spectrometry-based assays are regarded as the golden standard. Also, the design of the previous studies could explain these discrepancies with our findings. Notably, most studies were underpowered (n<20) and/or are not prospectively designed, which could have introduced additional variation and bias in the data. Yet another explanation could be that our results are affected by variability in BMI. Although previous studies have demonstrated a relationship between BMI and circulating sex steroid levels (45-48), we did not find differences between

baseline BMI and post-RRSO BMI or correlations between changes in BMI and changes in serum sex steroid levels.

In addition, we investigated differences in serum sex steroid levels between postmenopausal women before RRSO (natural menopause, T0) and premenopausal women at six weeks (T1) and seven months (T2) after RRSO (surgically induced menopause). We found higher serum estrogen levels in postmenopausal women before RRSO compared to the premenopausal group with surgical induced menopause after RRSO, although no differences for testosterone and androstenedione. For testosterone, these results were in line with one previous study (49), while another study found different results (50). A possible explanation could be that residual testosterone production, eg. in the adrenal glands, slowly declines with increasing age as previously has been described (44, 51). The difference in serum estrogen levels between naturally menopausal and surgically menopausal women is not in line with a previous study comparing naturally and surgically postmenopausal women warranting further research into possible underlying mechanisms (49).

Our second aim was to examine the relationship between changes in steroid levels and changes in menopausal symptoms and sexual functioning. Our results suggest that in premenopausal women, larger decreases in estrogens are associated with a worsening of sexual functioning seven months after RRSO. Furthermore, in postmenopausal women, larger decreases in androgens were associated with worsening of menopausal complaints (HFRSsum scores, ΔT0-T2) and sexual functioning (SFQ Arousal L, ΔT0-T1), although the latter association was not observed at ΔT0-T2. In addition, a decrease in estrogen level was associated with worsening sexual functioning (SFQ Orgasm), albeit only at ΔT0-T1. Notably, additional lowering of testosterone levels in postmenopausal women undergoing RRSO could therefore intensify menopausal complaints after seven months and shortly (at six weeks) decrease sexual functioning. Therefore, these results suggest that postmenopausal women might benefit from testosterone supplements after RRSO. Notably, testosterone supplements are not available in Europe anymore for postmenopausal women experiencing problems with their sexual functioning. Experts increasingly urge on its potential clinical benefits (52, 53), although it should be mentioned that caution should be taken with unwanted aromatization of testosterone to E2 in women with high breast cancer risk (eg. BRCA1/2 mutant carriers).

While several studies have been performed investigating the impact of sex steroid supplements on sexual functioning and menopausal complaints after RRSO (14-17), only few studies investigated the relationship between steroid levels and sexual functioning questionnaire scores (21, 54). In these studies, no relationship was found between serum

sex steroids and sexual functioning after RRSO. The difference in findings could be explained by the used approach to investigate these associations. Specifically, multivariable regression and logistic regression were applied to predict the sexual discomfort score or sexual dysfunction. Furthermore, different questionnaires were used to assess sexual functioning, i.e. the Female Sexual Function Index, the Sexual Activity Questionnaire and the Female Sexual Distress Scale Revised (55-57). Another factor could be the study design, which was cross-sectional instead of the cohort design in the present study. Also, the included women were grouped by sexual activity, not by menopausal status. While both approaches are statistically valid, this could have led to different outcomes.

Interestingly, no association between serum estradiol levels and HFRS-sum was detected and thus, our findings were not in line with current clinical practice advising estrogen supplements to relieve menopausal complaints. This advice is based on multiple clinical studies investigating the efficacy and safety of estrogen supplements in postmenopausal women (17, 58). While reports of associations between serum estradiol levels and the intensity of menopausal complaints remain scarce, the SWAN study, a large clinical study investigating over 3,000 women in menopause transition, found that low circulating estradiol levels were associated with a higher prevalence of hot flashes (59). This effect, however, was marginal, which could explain the absence of this association in our small cohort.

Some limitations were associated with our study. Firstly, the time of blood withdrawal was not standardized. Serum steroid hormone levels are known to fluctuate according to the circadian rhythm. This could have introduced variation into our data. To this end, we assessed whether sampling times before and after 11AM influenced serum sex steroid concentrations. We only found a significant difference for serum E1 levels in premenopausal women seven months after RRSO (Mann Whitney U, $p=0.02$) indicating variation introduced by time of blood collection was limited. Secondly, for postmenopausal women at baseline, one serum E2 level was abnormally high (622 pmol/L) indicating that this individual 1) was actually premenopausal, 2) received estrogen supplementation, 3) had an underlying E2 secreting tumor or, 4) pre-analytical or analytical errors were made. Although LH and FSH were within postmenopausal ranges, this individual had amenorrhea for five years and did not report estrogen supplementation intake, other factors explaining this high serum E2 level cannot be excluded. Thirdly, participants were not screened for other treatments influencing steroidogenesis, such as corticosteroids and aldosterone antagonists. Although our results do not indicate treatment with these therapies, this could potentially influenced serum sex steroid levels. Fourthly, p-values in our correlation analysis were not adjusted for multiple hypothesis testing and could have resulted in type II errors. To reduce the prevalence of

these errors, our investigation focused on questionnaire scores that correlated with both androgens or estrogens. Lastly, arguably marital status influences sexual functioning, which could have biased our results. However, we found that only SFQ pain scores were affected by marital status in premenopausal women six weeks after RRSO and in postmenopausal women at baseline and seven months after RRSO. This indicates that although there may have been some bias introduced in our analysis from differences in marital status, appears to be limited.

In conclusion, our results, derived from a longitudinal prospective cohort using state-of-the-art serum steroid assays, show that in premenopausal women, removal of the ovaries is accompanied by a decrease in serum androgens and estrogens both six weeks and seven months after RRSO. For estrogens, these decreases were associated with a decline in sexual functioning. In postmenopausal women, our findings show that only serum testosterone is decreased after RRSO, which indicates that postmenopausal ovaries maintain some testosterone production. Furthermore, in these women, our findings suggest that removal of the ovaries, together with a decrease in serum testosterone level, results in more menopausal complaints and a short term decline in sexual functioning. Therefore, these results support the Global Consensus Position Statement on the Use of Testosterone Therapy for Women (53), although further studies are warranted confirming these results.

Acknowledgements

None.

Disclosure Statement

The authors have nothing to disclose.

Data availability

The dataset that was established from the clinical trial data to produce the results of the present study is not publicly available. The dataset can be accessed upon reasonable request.

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Figure 1. Flow chart displaying participant inclusion and exclusion.

RRSO = risk-reducing salpingo-oophorectomy

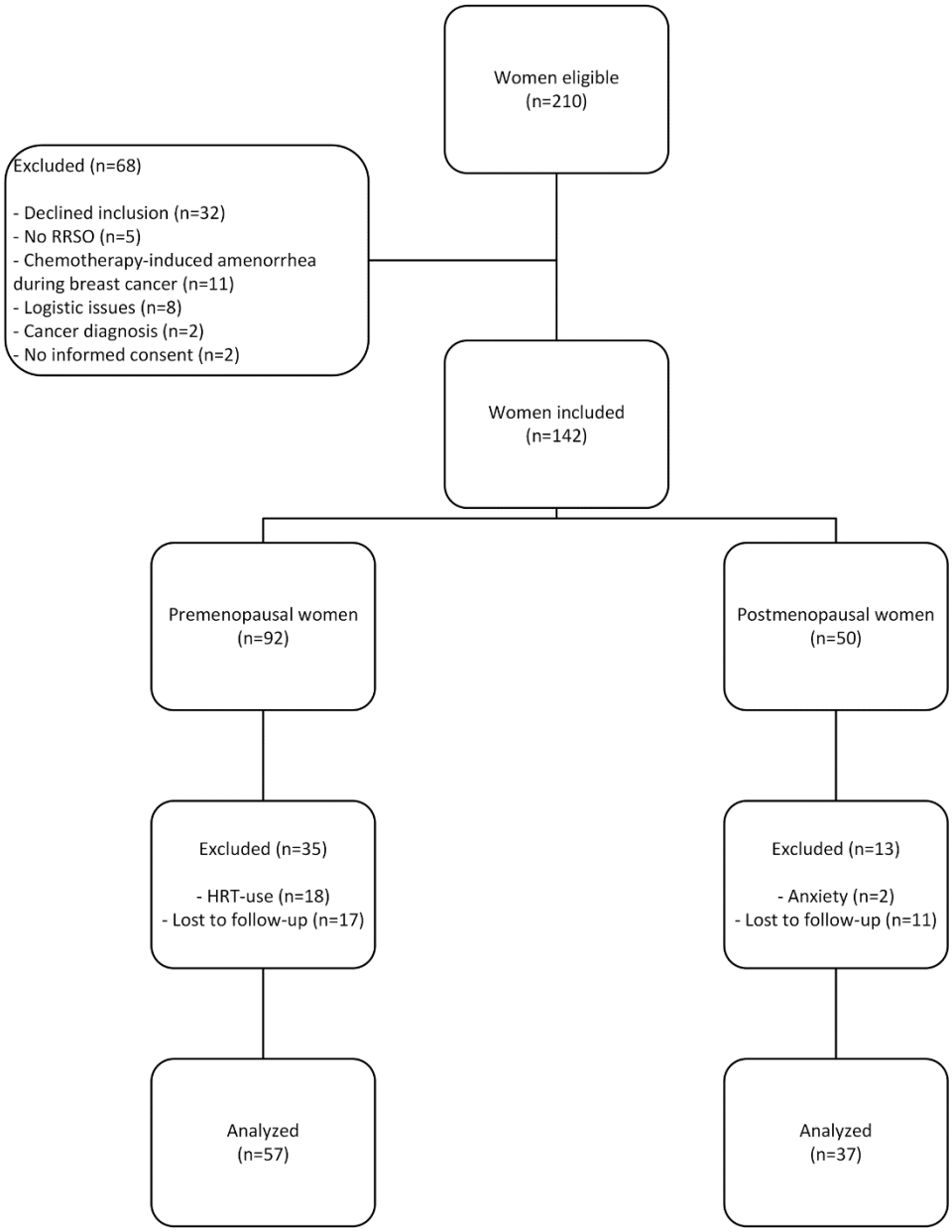

Figure 2. Box plot with log-normal transformed data for testosterone (A), androstenedione (B), E2 (C) and E1 (D) at each follow-up moment. Data was stratified for menopausal status (Black, premenopausal; Gray, postmenopausal). In case repeated measures ANOVA was performed, details are listed above the graph (*F*, F-statistic; p, p-value; η_g^2 , generalized eta-squared). Significant pair-wise comparisons are indicated with * symbols (*, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001).

ANOVA = analysis of variance

E1 = estrone

E2 = estradiol

T0 = Baseline

T1 = Six weeks

T2 = Seven months

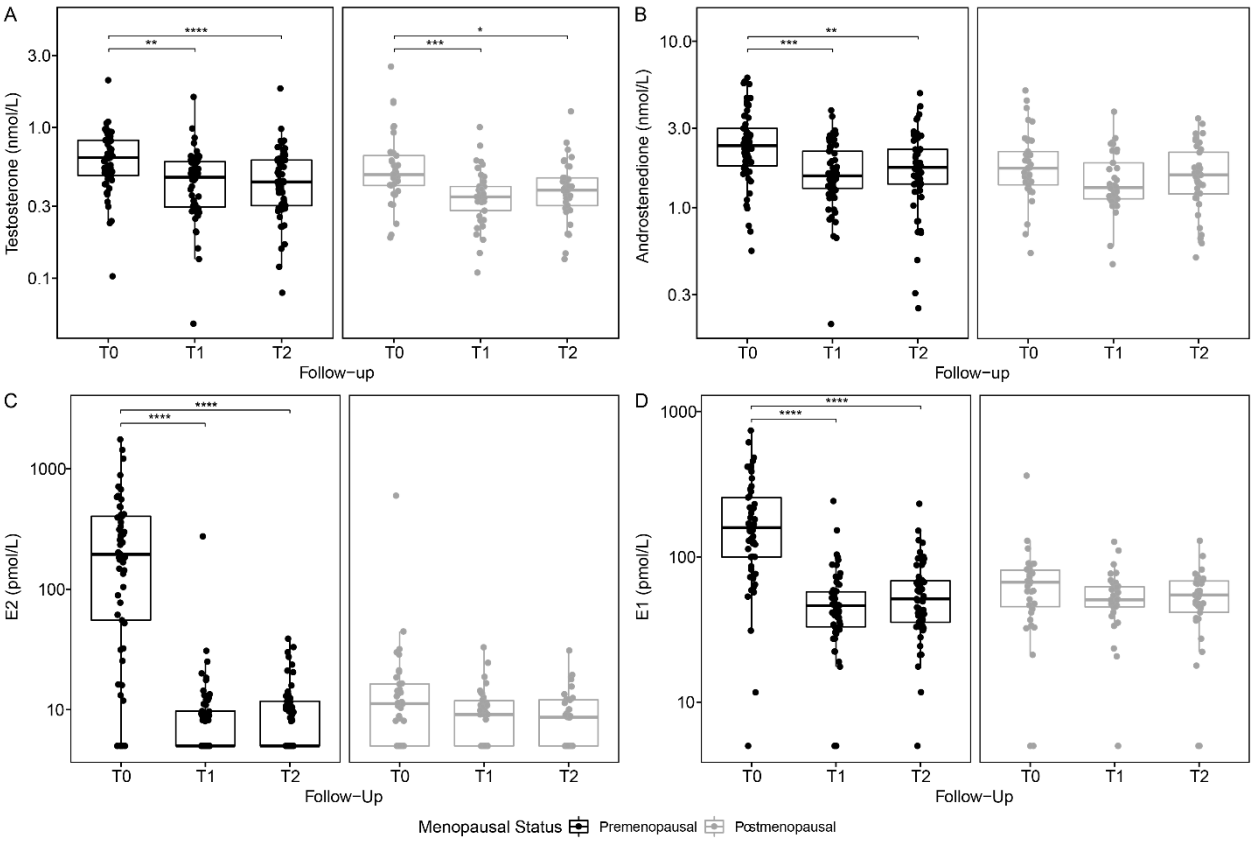

Figure 3. Waterfall plots showing relative individual changes between baseline and six weeks or seven months after RRSO.

E1 = estrone

E2 = estradiol

RRSO = Risk-reducing salpingo-oophorectomy

T0 = Baseline

T1 = Six weeks

T2 = Seven months

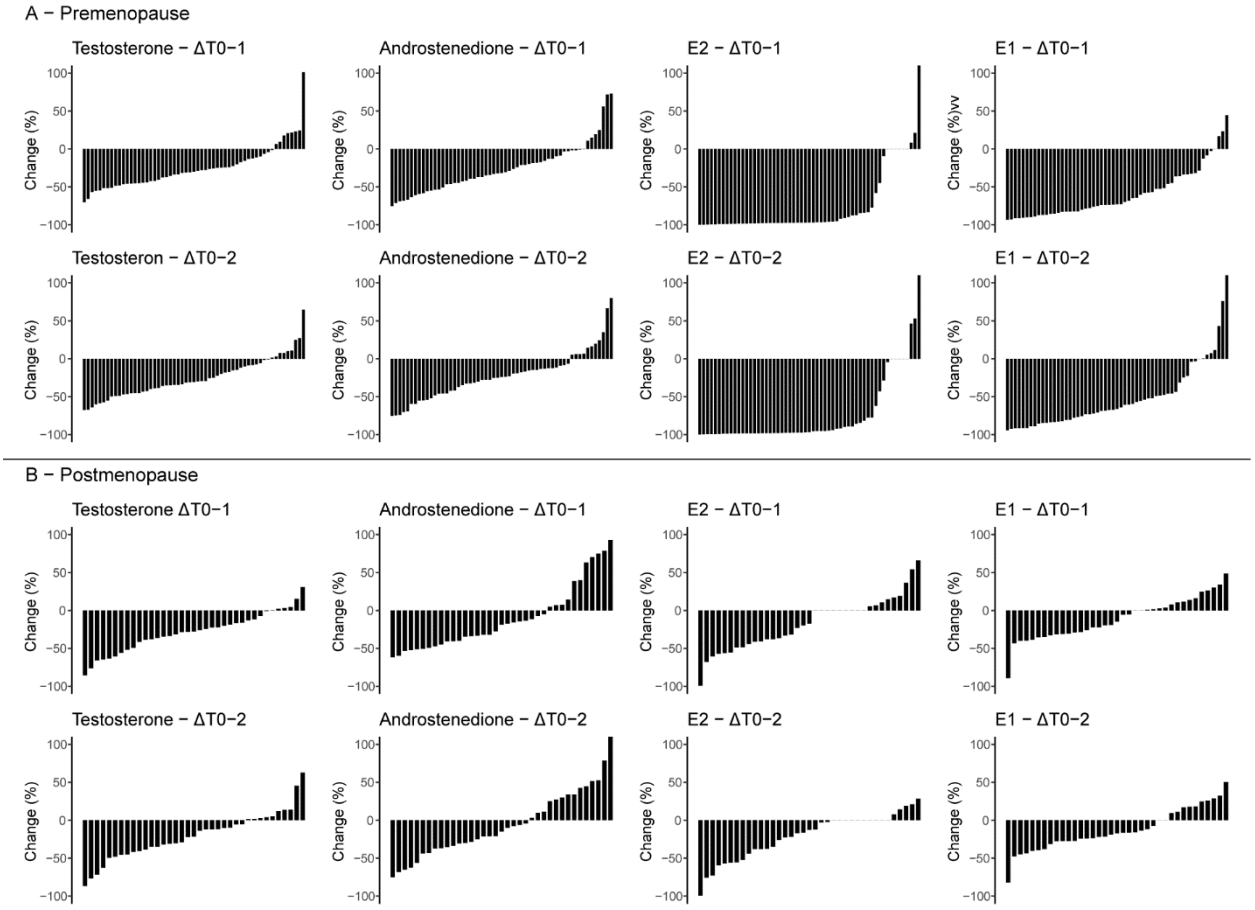
A − Premenopause
Testosterone − ΔT0−1
Androstenedione − ΔT0−1
E2 − ΔT0−1
E1 − ΔT0−1
Testosteron − ΔT0−2
Androstenedione − ΔT0−2
E2 − ΔT0−2
E1 − ΔT0−2
B − Postmenopause
Testosterone ΔT0−1
Androstenedione − ΔT0−1
E2 − ΔT0−1
E1 − ΔT0−1
Testosterone − ΔT0−2
Androstenedione − ΔT0−2
E2 − ΔT0−2
E1 − ΔT0−2
Change (%)
100
50
0
−50
−100

Figure 4. Box plots for comparison of naturally postmenopausal women (Black) and surgically postmenopausal women (Gray). Graphs for testosterone (A), androstenedione (B), E2 (C) and E1 (D) are displayed. Significant pair-wise comparisons are indicated with * symbols (*, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001).

E1 = estrone

E2 = estradiol

T0 = Baseline

T1 = Six weeks

T2 = Seven months

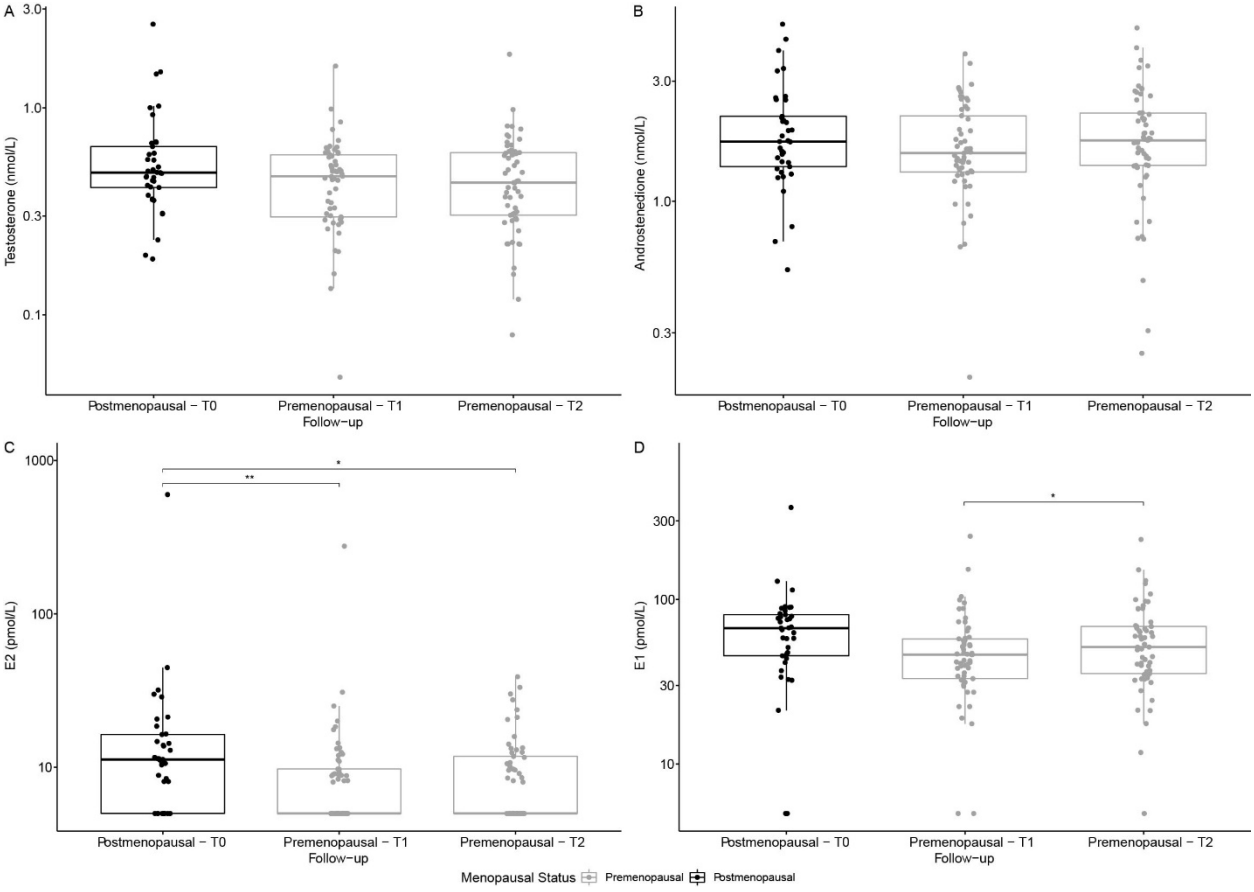

Figure 5. Box plots for FACTtotal (A), HFRSsum (B), SFQ Desire (C), SFQ Arousal L (D), SFQ Arousal S (E) and SFQ Orgasm (F) scores at each follow-up moment after RRSO. Data was stratified for menopausal status (Black, premenopausal; Gray, postmenopausal). Significant pair-wise comparisons are indicated with * symbols (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$).

E1 = estrone

E2 = estradiol

FACT = Functional assessment of cancer therapy

HFRS = Hot Flush Rating Scale

L = Lubrication

SFQ = Sexual functioning questionnaire

S = Sensation

T0 = Baseline

T1 = Six weeks

T2 = Seven months

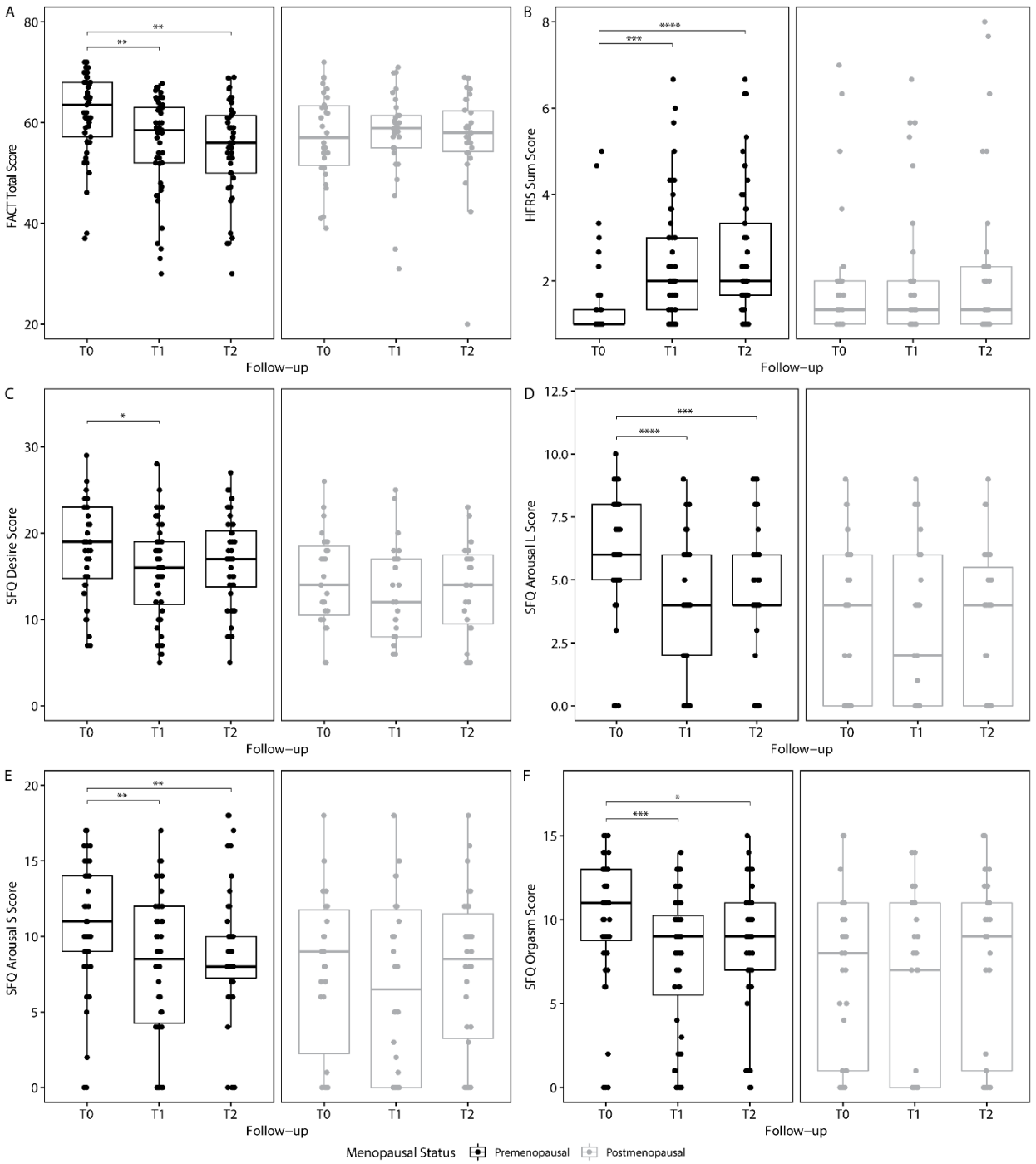
A
FACT Total Score
80
60
40
20
**
**
T0
T1
T2
Follow−up
B
HFRS Sum Score
8
6
4
2
****
***
C
SFQ Desire Score
30
20
10
0
*
D
SFQ Arousal L Score
12.5
10.0
7.5
5.0
2.5
0.0
***
****
E
SFQ Arousal S Score
20
15
10
5
0
**
**
F
SFQ Orgasm Score
15
10
5
0
*
***
Menopausal Status
Premenopausal
Postmenopausal

Figure 6. Spearman rho correlation matrix for correlations between changes in sex steroid levels and questionnaire scores. Correlation coefficients are color-coded at three levels from -1 (Blue) to 0 (White) to 1 (Red). Spearman's correlation coefficients that are significantly different from 0 (α=0.05) are indicated with * symbols (*, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001).

E1 = estrone

E2 = estradiol

FACT = Functional assessment of cancer therapy

L = Lubrication

SFQ = Sexual functioning questionnaire

S = Sensation

T0 = Baseline

T1 = Six weeks

T2 = Seven months

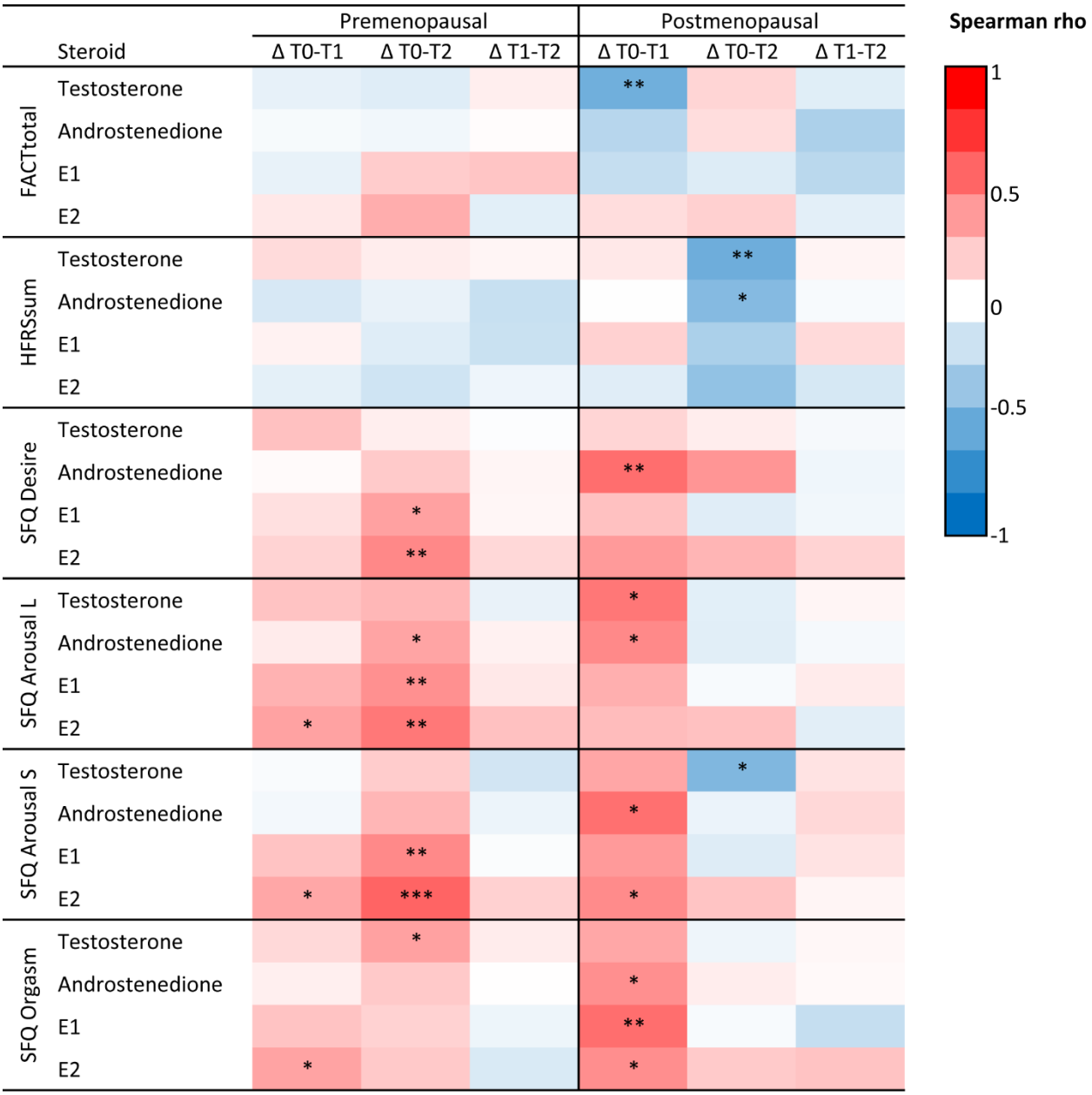
Premenopausal
Postmenopausal
Steroid
Δ T0-T1
Δ T0-T2
Δ T1-T2
Δ T0-T1
Δ T0-T2
Δ T1-T2
FACTtotal
Testosterone
Androstenedione
E1
E2
HFRSsum
Testosterone
Androstenedione
E1
E2
SFQ Desire
Testosterone
Androstenedione
E1
E2
SFQ Arousal L
Testosterone
Androstenedione
E1
E2
SFQ Arousal S
Testosterone
Androstenedione
E1
E2
SFQ Orgasm
Testosterone
Androstenedione
E1
E2
Spearman rho
1
0.5
0
-0.5
-1

Table 1. Baseline characteristics.

BMI = Body mass index

BC = Breast cancer

IQR = Interquartile range

n = Number

OC = Ovarian cancer

SD = Standard deviation

T0 = Baseline

	Premenopausal	Postmenopausal	p-value	Overall p-value
n (%)	57 (61)	37 (39)		
Age, year - mean (SD)	44 (4)	57 (6)	<0.0001	
BMI, kg/m^2 - median (IQR)	23.2 (22.0-25.7)	25.0 (22.3-27.5)	0.09	
Marital Status - n (%)				0.73
Married/cohabitating	49 (86)	30 (81)		
Unmarried/ without partner	8 (14)	7 (19)		
Parity - n (%)				0.26
None	10 (18)	11 (30)		
≥ one	47 (82)	26 (70)		
History of breast cancer - n (%)				0.51
Yes	21 (37)	16 (43)		
No	36 (63)	21 (57)		
DNA status - n (%)				0.01
BRCA 1/2 carrier	47 (83)	20 (54)		
Negative	6 (11)	12 (32)		
Unknown	4 (6)	5 (14)		
Comorbidities - n (%)				0.05
Pulmonary disease	0 (0)	3 (8)		
Cardiac disease	0 (0)	0 (0)		
Hypertension	2 (4)	2 (5)		
Stroke	0 (0)	0 (0)		
Renal disease	0 (0)	0 (0)		
Diabetes	0 (0)	0 (0)		
Arthralgia	5 (9)	8 (22)		
Psychological problems	3 (5)	0 (0)		
Malignancies (Ex BC and OC)	2 (4)	0 (0)		
None/Unknown	45 (79)	24 (65)		
Days from T0 to RRSO - median (IQR)	3 (2-7)	4 (2-7)	0.89	

Chapter 5 – General discussion

Since the emergence of liquid chromatography-tandem mass spectrometry (LC-MS/MS) in the medical laboratory, accurate measurement in various diagnostic niches has been enabled (1). However, recognition of its capabilities by clinicians has been limited, as inferior analytical methods are still applied in major clinical trials (**Chapter 1.1**) (2-4). It is therefore essential that assay performance differences potentially affecting clinical decision-making or study results are increasingly revealed in relevant patient populations to optimize current and future diagnostics.

The aim of this thesis was first to describe the development and validation of blood-based steroid LC-MS/MS assays and, subsequently, reveal important technical discrepancies between LC-MS/MS assays and relevant study populations. Finally, the steroid LC-MS/MS assays are used in advanced prostate cancer (PCa) patients and women undergoing oophorectomy to explore their clinical utility and possible diagnostic purposes.

LC-MS/MS assay development and validation

The blood-based testosterone (**Chapter 1.2 and 1.3**) and estrogen (**Chapter 1.5**) LC-MS/MS assays in this thesis were designed for clinical application in castrated PCa patients and postmenopausal women, respectively (5-7). The assay was able to quantitate testosterone in all samples obtained from castrated prostate cancer patients treated with or without enzalutamide. In addition, an LC-MS/MS assay for the simultaneous measurement of estrone (E1) and estradiol (E2) in human serum was developed and validated. In all samples from healthy men and women, E1 levels could be quantitated, whereas in 25% of postmenopausal samples, E2 levels were below the lower limit of quantitation. Furthermore, an upper limit of the reference interval could be determined for both E1 and E2 in all groups and a significant difference between females aged below 41 and above 60 was observed.

In literature, blood-based quantitation of testosterone by LC-MS/MS has been extensively described. Major differences in performance generally depend on the generation of the liquid chromatography (LC) and mass spectrometry (MS) instrument (5, 6, 8). Other methods described to enhance testosterone LC-MS/MS assay performance are application of alternative ionization chambers or chemical derivatization (9-11). For testosterone, atmospheric-pressure chemical ionization and atmospheric-pressure photoionization have been used instead of electrospray ionization (ESI) to increase ionization efficiency of testosterone due to their high suitability for highly apolar compounds, such as steroids (12, 13). However, for medical laboratories, it is preferable to avoid manual switching of ionization sources and to maintain an ionization source applicable for a broader range of polarity (i.e.

ESI). In addition, chemical derivatization increases ionization efficiency of testosterone MS analysis by formation of oximes, hydrazones or by esterification. Potentially, chemical derivatization can increase sensitivity of LC-MS/MS assays substantially, although a potential drawback is the formation of structural isomers, which can influence chromatographic separation and thus specificity greatly (14). Notably, in this thesis, measurement of testosterone without derivatization and application of ESI provided sufficient sensitivity to produce accurate results for virtually all castrated PCa patients.

In **Chapter 1.5**, this thesis also describes the development and validation of an LC-MS/MS assay for the quantitation of estradiol (E2) and estrone (E1) in human serum. In comparison to testosterone, blood-based estrogen LC-MS/MS assays are less prevalently described in literature. Probably, this is due to the aromatization of the left carbohydrate ring leading to molecular stabilization and a decrease of ionization efficiency for the attached hydroxyl group. Furthermore, a high sensitivity is required for E2 to quantitate the lower picomolar range in postmenopausal women and men. Methods to increase sensitivity include chemical derivatization, which is primarily performed with dansyl chloride, and two-dimensional liquid chromatography (15-17). Considerably high sensitivities can be achieved with these procedures, although sample work-up is laborious and prone to errors making this approach not preferable for medical laboratories. An easier approach is the addition of ammonium fluoride (NH4F) to the mobile phase, which has a high proton affinity during ionization resulting in high negative ionization efficiency (18, 19). Taking into account its application in the medical laboratory, the estrogen LC-MS/MS assay reported in this thesis only incorporates the addition of NH4F for enhancement of the analytical sensitivity.

Method comparison

Blood-based measurement of testosterone and E2 in medical laboratories is still primarily performed with automated immunoassays (AIA). This thesis aimed to reveal relevant differences in quantitation between AIA and LC-MS/MS for testosterone and E2 in castrated PCa patients (**Chapter 2.2**) and postmenopausal women (**Chapter 1.5**), respectively. For testosterone, in 120 samples from castrated PCa patients, four commonly applied AIA all generated significantly higher results and Passing-Bablok regressions showed poor agreement between the testosterone AIA and the LC-MS/MS assay. Furthermore, relative differences were significantly higher than the desirable total error estimated for castrated PCa patients in the biological variation study (**Chapter 1.3**). For E2 analysis, relative differences up to 138% were observed in female samples. Notably, high relative differences were mainly observed at low concentrations (<200 pmol/L) characteristic for postmenopausal women.

In a clinical oncology setting, E2 levels are presently measured to monitor occurrence of ovarian function recovery in breast cancer patients with chemotherapy-induced amenorrhea treated with aromatase inhibitors. A falsely elevated E2 AIA result could have serious clinical implications as physicians could potentially choose for an unnecessary alternative treatment strategy. However, evidence demonstrating the clinical impact of E2 AIA shortcomings for these patients has yet to be presented. Therefore, further investigation is warranted before E2 LC-MS/MS assays can be clinically implemented for this setting.

For advanced PCa patients, testosterone levels are measured to monitor castration adequacy and castration-resistance. According to clinical guidelines, testosterone levels should remain below the castration cut-off set at 1.7 nmol/L. This cut-off is based on historical consensus and a post-hoc analysis of the PR-7 trial (2, 20, 21). Although the evidence for this cut-off seems to be apparent, there has been some debate on its validity. Early investigations into the pharmacodynamics of surgical and chemical castration applied historical assay techniques to measure circulating levels of testosterone, such as double isotope derivative assays (22-24). In general, these assays were inaccurate and had low sensitivities. It is probable the 1.7 nmol/L cut-off originates from these investigations as guidelines refer to a consensus established in that era and at least one study reported values < 1.7 nmol/L. Later studies incorporating AIA suggested a lower castration cut-off value. Already in 2000, one study estimated population intervals of 35 surgically castrated PCa patients using an AIA and found a maximum of 1 nmol/L and referred to a recommended cut-off at 0.7 nmol/L (25). This recommended cut-off value was later confirmed by two clinical studies, in which lower testosterone levels were associated with improved treatment outcome (2, 26).

Still, the results of these studies did not lead to a lower castration cut-off definition in clinical guidelines. Although the reason is not specified, it is apparent from this thesis that AIA occasionally detect testosterone levels > 0.7 nmol/L in adequately castrated PCa patients. As assay differences are not widely recognized by clinicians, erroneous decisions on treatment strategy could be easily made for castrated PCa patients with falsely elevated AIA testosterone results. To further increase awareness, it is vital that such differences are increasingly highlighted in clinical studies.

Another issue is how to appropriately classify patients as inadequately castrated. Originally, the castration cut-off was defined at 1.7 nmol/L using historical double isotope derivative assays in surgically castrated PCa patients (22, 23). Subsequently, chemically castrated PCa patients with testosterone levels above this cut-off value were categorized as inadequately castrated. However, as demonstrated in this thesis, a more accurate castration cut-off, as

determined with an LC-MS/MS assay, is highly likely to be substantially lower than 1.7 nmol/L. In light of a probably lower castration cut-off, it should be noted that testosterone levels associated with inadequate castration have yet to be identified and it remains questionable whether this can be achieved. As shown in the biological variation study of this thesis, testosterone levels have a high individuality in castrated PCa patients and remain relatively close to the intra-individual mean concentration. This would suggest a more personalized approach in evaluating castration adequacy, although this would require monitoring castration levels over an extended time period.

Clinical utility of steroid LC-MS/MS assays

Finally, this thesis aimed to investigate the clinical utility of blood-based androgen and estrogen LC-MS/MS assays. In PCa patients, testosterone levels measured by LC-MS/MS during chemical castration alone (hormone-sensitive prostate cancer, HSPC, **Chapter 2.3**) or in combination with enzalutamide (castration-resistant prostate cancer, CRPC, **Chapter 2.4**) were investigated as an independent risk factor. For HSPC patients, levels above the median were associated with shorter times to progression. In patients that progressed to CRPC and received first line enzalutamide, the 25% lowest testosterone levels were associated with shorter progression-free survival (PFS). These cohorts were analyzed retrospectively.

Notably, in this thesis we described that for HSPC patients lower testosterone levels were associated with a PFS benefit when treated with androgen-deprivation therapy, while for CRPC patients higher testosterone levels were associated with better PFS when treated with second line enzalutamide treatment. Similar observations in other CRPC treatment cohorts, albeit applying AIA, have been reported for HSPC and CRPC patients (2, 27-29). For HSPC, the therapeutic objective of chemical castration is depletion of gonadal testosterone, which is regarded as a primary driver of PCa growth. In this context, testosterone levels should be minimized and kept at the lowest possible concentration. Conversely, in a castration-resistant state, high residual levels of testosterone indicate that the tumor is still hormone sensitive and patients generally have a good response on additional hormonal therapy with androgen receptor targeted agents (ARTA, eg. Abiraterone and enzalutamide). This has been substantiated by previous studies suggesting the sensitization to low androgen levels and *de novo* androgen synthesis as resistance mechanisms (30-33). Although these studies cannot be directly linked to the serum testosterone measurements, they are consistent with our findings.

This thesis and other previous studies indicate a role for testosterone as an independent risk factor for advanced PCa treatment. However, it is apparent some hurdles prevent its clinical implementation, such as the rapidly changing treatment landscape. Following the results of a

multitude of randomized-controlled trials (eg. STAMPEDE, CHAARTED, GETUG, PEACE-1, ARASENS), in which survival benefit was demonstrated by combining standard-of-care with additional ARTA in mHSPC patients, new treatment strategies are being implemented in clinical care (34-39). As Chapter 8 demonstrates that patients with high castration testosterone levels have a shorter time to CRPC, it is probable that the improved survival of additional ARTA in mHSPC treatment can be explained by this subset of patients. Potentially, LC-MS/MS testosterone measurement could identify which patients benefit from additional ARTA during mHSPC treatment. Although pharmaceutical companies marketing the new ARTA will probably not favor this approach, it can be valuable for patients and it is therefore essential that standard testosterone LC-MS/MS analyses are included in the ongoing randomized-controlled trials or is investigated in post-hoc analyses of previous large trials.

In addition, this thesis aimed to prospectively research the relationship between changes in testosterone, androstenedione, E2 and E1 levels and menopausal complaints in women undergoing risk-reducing salpingo-oophorectomy (RRSO)(**Chapter 3.1**). Herein, decreases in estrogens were associated with a decrease in sexual functioning for premenopausal women. Furthermore, in postmenopausal women, testosterone levels were decreased after RRSO indicating that postmenopausal ovaries retain some testosterone production. Notably, this decrease in testosterone levels was associated with an increase in menopausal complaints.

While the relationship between sex steroid levels and menopausal complaints has been suggested and investigated previously, the results have not been clear-cut (40-43). Some limitations of previous studies include study design (i.e. retrospective) and application of immunoassays for the blood-based measurement of sex steroids. To address these limitations, this relationship was investigated in a longitudinal prospective study, in which LC-MS/MS assays were applied for the measurement of sex steroids. Arguably, these points contribute to the strengths of the presented evidence, although it should be mentioned a larger sample size and adjustment for type II errors would be necessary to make definitive conclusions on this topic.

However, the findings support the Global Consensus Position Statement on the Use of Testosterone Therapy for Women (44). In this statement, physicians call for the administration of testosterone supplements in postmenopausal women experiencing menopausal complaints. This could potentially alleviate symptoms in these women increasing their overall quality of life. In this case, it would be important to caution for supraphysiological levels of testosterone in these women as high aromatization rate to E2

could increase breast cancer risk. Therefore, it would be essential to monitor testosterone, and arguably E2, levels to minimize this risk. Notably, this should include measurement by LC-MS/MS to prevent unnecessary medical action due to falsely elevated AIA results.

Concluding remarks

Since the introduction of LC-MS/MS in the medical laboratory, some medical fields have noticed the discrepancies with AIA and embraced this superior assay technique. For hormonal treatment in advanced PCa and postmenopausal women, steps toward this direction have been made, although we are not quite near the finish line. To achieve a leap forward, an increased effort towards collaboration between medical laboratory and clinicians is vital and we are optimistic this thesis contributes to this ultimate goal.

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Summary

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is a powerful tool for various diagnostic niches in the medical laboratory. Although some clinical fields apply this technique for diagnostic purposes, this has not been established for sex steroid analysis in castrated prostate cancer (PCa) patients and postmenopausal women. Furthermore, enabling higher sensitivities and accuracies with LC-MS/MS can reveal new clinical applications. This thesis addressed the development and validation of blood-based sex steroid LC-MS/MS assays. Subsequently, these assays were compared with commonly applied automated immunoassays (AIA) and potential clinical utilities were investigated in advanced PCa patients and women undergoing oophorectomy.

Section 1 primarily describes the development and validation of blood-based sex steroid LC-MS/MS assays and opens with a letter emphasizing the necessity of adequately reporting the analytical method in clinical trials (**Chapter 1.1**). **Chapter 1.2** details a protocol for the measurement of testosterone in human serum by LC-MS/MS. For this method, testosterone was measured in 5 minutes using a 5 µm particle size C18 column and an API4000 (AB Sciex) mass spectrometry (MS) instrument. The lower limit of quantitation (LLOQ) was determined at 0.17 nmol/L and total imprecision was ≤ 4.7% coefficient of variation (CV). In **Chapter 1.3**, biological variation (BV) of serum testosterone levels was estimated in PCa patients treated with chemical castration with or without enzalutamide. Testosterone was measured using a newly developed and validated LC-MS/MS assay. A higher quality column (1.7 µm core shell C18) and MS instrument (QTRAP6500+, AB Sciex) allowed for increased sensitivity (LLOQ, 0.025 nmol/L). BV was similar between groups and notably, within-patient variation was considerably lower than the between-patient variation indicating a high individuality. **Chapter 1.4** highlights the openly available R-script that was used for BV estimation in Chapter 4 and discusses its necessity in the context of modern BV studies. **Chapter 1.5** describes an LC-MS/MS assay for the routine quantitation of estrone (E1) and estradiol (E2) in human serum and compares results obtained in healthy male and female volunteers with an in-house E2 AIA. This assay is able to quantitate E1 and E2 levels as low as 6.9 and 8.0 pmol/L, respectively. Notably, below 200 pmol/L agreement between the E2 LC-MS/MS assay and the AIA was poor and relative differences up to 138% were observed. To conclude, this chapter highlights relevant differences between LC-MS/MS and an AIA, as well as performance differences between individual LC-MS/MS assays. Although not all E2 levels could be quantitated in healthy men and women, sensitivity was 10 times higher than the E2 AIA and the method allowed quantitation of E1 in all healthy volunteer samples.

Importantly, for all castrated PCa patients, testosterone could be quantitated and biological variation was for the first time determined in this population.

Section 2 focuses on testosterone analysis in PCa patients and describes studies investigating technical differences between assays and potential clinical applications. In **Chapter 2.1**, a detailed review on the history, current situation and perspectives of testosterone analysis in PCa patients is provided. Specifically, the literature review discusses the evolution of hormonal treatment in PCa; from the discovery of PCa androgen dependence and the first surgical castration to more recently developed androgen receptor and CYP17A1 inhibitors. Subsequently, the review discusses the different assay techniques that have been used throughout the years and particularly focuses on their strengths and shortcomings. Finally, potential clinical utilities are listed and evaluated, such as the predictive and prognostic value of testosterone analysis in hormone-sensitive and castration-resistant PCa patients. Based on this information, clinical and research recommendations are made. **Chapter 2.2** investigates differences between the testosterone LC-MS/MS assay and four commonly applied testosterone AIA in hormone-sensitive PCa patients treated LHRH agonist monotherapy. We demonstrate that, in comparison with the LC-MS/MS assay, all AIA generate significantly different testosterone results and all AIA show poor agreement. Furthermore, relative mean differences between all AIA and the LC-MS/MS assay exceeded the desirable total error determined in castrated PCa patients. Importantly, these results suggest that a lower castration cut-off is more appropriate for evaluation of castration adequacy in PCa patients. **Chapter 2.3** studies on-treatment testosterone levels measured by LC-MS/MS as an independent risk factor for PCa patients treated with LHRH agonists or antagonists. Herein, testosterone levels equal or below the median were associated with improved progression-free survival (PFS). Notably, no PFS benefit was observed for high testosterone AIA results. **Chapter 2.4** investigates the predictive value of during- and pretreatment LC-MS/MS testosterone levels in castration-resistant PCa patients treated with first line enzalutamide. In contrast to the results observed in hormone-sensitive PCa, the 25% highest testosterone levels, for both during and pretreatment samples, were associated with improved PFS. While the evidence obtained in this chapter does not justify adjustment of clinical guidelines, it does underline the added clinical value of blood-based testosterone LC-MS/MS measurements in castrated PCa patients and warrants its incorporation in large ongoing clinical trials.

Section 3 shifts the focus to the analysis of multiple sex steroids in women with a high risk of ovarian cancer. For these women, an important preventive measure is the surgical removal of the ovaries, also called risk-reducing salpingo-oophorectomy (RRSO). For premenopausal women, RRSO causes the immediate onset of the menopause transition, which is commonly

accompanied by menopausal complaints, such as hot flashes and sexual dysfunction. Notably, these symptoms have previously been ascribed to changes in sex steroid levels. **Chapter 3.1** prospectively investigates the relationship between changes in sex steroid levels and menopausal complaints in pre- and postmenopausal women undergoing RRSO. For premenopausal women, a decrease in estrogens was associated with a decrease in sexual functioning and, for postmenopausal women, a decrease in testosterone was associated with a decrease in hot flash frequency. Notably, these findings suggest a potential role for steroid supplementation for symptom mitigation in women suffering from menopausal complaints after RRSO.

In summary, it is evident that LC-MS/MS assays offer increased performance in terms of sensitivity and specificity over their AIA counterparts. Importantly, major differences in generated results between these assay techniques are previously observed in populations and concentration ranges relevant for clinical decision-making and study outcomes. Furthermore, additional clinical utilities can be enabled with LC-MS/MS. This thesis describes work on these points for castrated PCa patients and women at risk of ovarian cancer and concludes that application of sex steroid LC-MS/MS assays probably optimizes routine diagnostics for these populations. It is therefore essential that these results are translated into concrete changes for routine diagnostics through follow-up clinical studies.

Samenvatting

Vloeistof chromatografie-tandem massa spectrometrie (LC-MS/MS) is een krachtig analytisch hulpmiddel voor verschillende diagnostische niches in het medische laboratorium. Hoewel sommige klinische velden deze techniek al gebruiken voor diagnostische doeleinden, is dit nog niet het geval voor de analyse van geslachtshormonen in gecastreerde prostaatkanker (PKa) patiënten en postmenopauzale vrouwen. Bovendien kan een hogere sensitiviteit en nauwkeurigheid met behulp van LC-MS/MS nieuwe klinische toepassingen onthullen. Deze thesis behandelt de ontwikkeling en validatie van bloed gebaseerde geslachtshormoon LC-MS/MS bepalingen. Deze bepalingen zijn vergeleken met algemeen gebruikte geautomatiseerde immunoassays (AIA) en vervolgens zijn potentiële klinische toepassingen onderzocht in geavanceerde PKa patiënten en vrouwen die een ovariëctomie ondergaan.

Sectie 1 beschrijft hoofdzakelijk de ontwikkeling en validatie van bloed gebaseerde geslachtshormoon LC-MS/MS bepalingen en opent met een brief, die de noodzakelijkheid van het adequaat rapporteren van de analytische methode in klinische studies benadrukt (**Hoofstuk 1.1**). **Hoofdstuk 1.2** somt een protocol op voor het meten van testosteron met behulp van LC-MS/MS in humaan serum. In deze methode wordt testosteron in 5 minuten met een 5 µm deeltjesgrootte C18 kolom en een API4000 (ABSciex) massa spectrometer (MS) gemeten. De lower limit of quantitation (LLOQ) is vastgesteld op 0.17 nmol/L en de totale imprecisie is ≤ 4.7% coefficient of variation (CV). In **Hoofdstuk 1.3** is de biologische variatie (BV) van serum testosteron geschat voor PKa patiënten, die behandeld worden met chemische castratie met of zonder toevoeging van enzalutamide. Testosteron is gemeten met een nieuw ontwikkelde en gevalideerde LC-MS/MS methode. Een hogere kwaliteit kolom (1.7 µm core shell C18) en MS (QTRAP6500+, AB Sciex) staat een verhoogde sensitiviteit toe (LLOQ, 0.025 nmol/L). BV is vergelijkbaar tussen de groepen en opmerkelijk is dat de binnen-patiënt variatie aanzienlijk lager is dan de tussen-patiënt variatie. Dit geeft aan dat testosteron waarden binnen een patiënt stabiel blijven ten opzichte van de tussen-patiënt waarden. **Hoofdstuk 1.4** beschrijft een LC-MS/MS bepaling voor de routinematige kwantificatie van oestron (E1) en oestradiol (E2) in humaan serum en vergelijkt de resultaten verkregen in gezonde mannen en vrouwen met een in-huis E2 AIA. Deze bepaling is in staat om E1 en E2 spiegeld respectievelijk zo laag als 6.9 en 8.0 pmol/L te kwantificeren. Opmerkelijk is dat er een slechte overeenstemming bestaat tussen de E2 LC-MS/MS bepaling en AIA was bij spiegels lager dan 200 pmol/L en dat er relatieve verschillen tot en met 138% geobserveerd zijn. Concluderend markeert dit hoofdstuk relevante verschillen tussen een LC-MS/MS bepaling en een AIA, net zoals verschillen in kwaliteit tussen

individuele LC-MS/MS bepalingen. Hoewel niet alle E2 spiegels gekwantificeerd konden worden in gezonde mannen en vrouwen, is de sensitiviteit 10 keer lager dan de E2 AIA en staat de bepaling E1 kwantificatie toe in alle monsters van gezonde vrijwilligers. Belangrijk is dat voor alle gecastreerde PKa patienten testosteron gekwantificeerd kon worden en dat BV voor het eerst vastgesteld is in deze populatie.

Sectie 2 focust zich op testosteron analyse in PKa patiënten en beschrijft studies die technische verschillen tussen bepalingen en potentiele klinische applicaties onderzoeken. In **Hoofdstuk 2.1** is een gedetailleerde review over de geschiedenis, huidige situatie en perspectieven van testosteron analyse in PKa patiënten beschreven. Specifiek bespreekt de literatuur studie de evolutie van hormoon behandeling in PKa; van de ontdekking dat PKa afhankelijk is van androgenen en de eerste chirurgische castratie tot de meer recent ontwikkelde androgeen receptor en CYP17A1 remmers. Vervolgens worden de verschillende bepalingstechnieken besproken die door de jaren heen zijn gebruikt, waarbij de sterke aspecten en tekortkomingen worden belicht. Daarnaast worden potentiele klinische toepassingen besproken, zoals de predictieve en prognostische en prognostische waarde van testosteron analyse in hormoon-sensitieve en castratie-resistente PKa patiënten. Gebaseerd op deze informatie zijn er onderzoeks- en klinische aanbevelingen gedaan. **Hoofdstuk 2.2** onderzoekt verschillen tussen de testosteron LC-MS/MS bepaling en vier algemeen toegepaste testosteron AIA in hormoon-sensitieve PKa patiënten, die behandeld worden met LHRH agonist monotherapie. We tonen aan dat, in vergelijking met de LC-MS/MS bepaling, alle AIA significant verschillende waarden genereren en dat er een slechte overeenkomst is. Bovendien overschreden de relatieve gemiddelde verschillen tussen alle AIA en de testosteron LC-MS/MS bepaling de wenselijke totale foutmarge, zoals bepaald in gecastreerde PKa patiënten. Belangrijk is dat deze resultaten suggereren dat een lagere castratie afkapwaarde meer geschikt is voor het evalueren van castratie adequaatheid. **Hoofstuk 2.3** bestudeert testosteronspiegels gemeten met behulp van LC-MS/MS tijdens LHRH agonist of antagonist behandeling van PKa patiënten als onafhankelijke risicofactor. Hierin zijn testosteronspiegels lager of gelijk aan de mediaan geassocieerd met een betere progressie-vrije overleving (PVO). Opmerkelijk is dat er geen PVO wordt geobserveerd voor hoge testosteron spiegels, die met een AIA gemeten waren. **Hoofdstuk 2.4** onderzoekt de predicatieve van testosteronspiegels gemeten met LC-MS/MS tijdens- en voor 1^{e} lijns enzalutamide behandelde castratie-resistente PKa patiënten. In tegenstelling tot de resultaten in hormoon-sensitieve PKa, zijn de 25% hoogste testosteronspiegels, voor zowel de metingen tijdens en voor enzalutamide behandeling, geassocieerd met een betere PVO. Hoewel het bewijs in dit hoofdstuk de aanpassing van klinische richtlijnen rechtvaardigt, onderstreept het wel de toegevoegde klinische waarde van bloed gebaseerde testosteron

LC-MS/MS metingen in gecastreerde PKa patiënten en rechtvaardigt zijn incorporatie in grote voortdurende klinische studies.

Sectie 3 verlegt de focus naar de analyse van meerdere geslachtshormonen in vrouwen met een hoog risico voor ovariumcarcinoom. Een belangrijke voorzorgsmaatregel is de chirurgische verwijdering van de eierstokken, ook wel profylactische salpingectomie genoemd (PSE). Voor premenopauzale vrouwen veroorzaakt dit de onmiddellijke start van de menopauze, welke algemeen vergezeld wordt met menopauzale klachten, zoals opvliegers en seksuele dysfunctie. Opmerkelijk is dat deze symptomen in voorgaande literatuur worden toegeschreven aan veranderingen in geslachtshormoonspiegels. **Hoofstuk 3.1** onderzoekt prospectief de relatie tussen veranderingen in geslachtshormoonspiegels en menopauzale klachten in pre- en postmenopauzale vrouwen, die een PSE ondergaan. Voor premenopauzale vrouwen is een verlaging in oestrogenen geassocieerd met een verlaging van het seksueel functioneren en voor postmenopauzale vrouwen is een verlaging in testosteron geassocieerd met een verlaging in de frequentie van opvliegers. Deze resultaten suggereren een potentiele rol voor geslachtshormoonaanvulling om symptomen te bestrijden in vrouwen die last hebben van menopauzale klachten na PSE.

Samenvattend is het evident dat LC-MS/MS bepalingen verhoogde prestatie bieden in termen van sensitiviteit en specificiteit ten opzichte van hun AIA tegenhangers. Belangrijk is dat in eerdere literatuur grote verschillen tussen deze bepalingstechnieken zijn geobserveerd in populaties en concentratie intervallen, die relevant zijn voor klinische beslissingen en studie uitkomsten. Bovendien kunnen er met LC-MS/MS potentiele klinische toepassingen beschikbaar gesteld worden. Deze thesis beschrijft werk aangaande deze punten voor gecastreerde PKa patienten en vrouwen met verhoogd risico op ovariumcarcinoom en concludeert dat applicatie van geslachtshormoon LC-MS/MS bepalingen waarschijnlijk de routinematige diagnostiek voor deze populaties kan verbeteren. Het is daarom essentieel dat deze resultaten vertaald worden in concrete veranderingen door het uitvoeren van klinische follow-up studies.

List of scientific publications

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Dankwoord

Het beeld dat bij promotieonderzoek overheerst, is dat het veelal een eenzame taak is. Hoewel dat voor een deel waar is, zijn samenwerkingen en de indirecte ondersteuning essentieel voor een goede afloop van je project. Dit was voor mij niet anders en, ondanks dat mijn dankbaarheid verder reikt dan deze woorden, wil ik dit hier toch kort benoemen.

Op de eerste plaats wil ik mijn copromotor, supervisor, en toch ook wel mentor, Dr. Huub van Rossum bedanken. Aan het einde van mijn eerste masterstage werd ik met Huub in contact gebracht voor een eventuele afstudeerstage bij het AVL en na een kennismakingsgesprek werd ik ingewijd in de wereld van de klinische chemie. Het zou het begin zijn van 6 fijne samenwerkingsjaren, waarin er naast de zekere tegenslagen vooral mooie momenten zijn geweest, en ik veel enthousiasme voor het vakgebied heb gekregen. Ik wil je enorm bedanken dat je mij het vertrouwen en de mogelijkheid hebt gegeven een nieuw LC-MS/MS systeem te beheren en daarvoor klinische bepalingen te ontwikkelen. Ik hoop sterk dat onze paden in de toekomst nog een keer kruisen.

Mijn promotoren, Prof. Dr. Sabine Linn en Prof. Dr. Henk van der Poel, wil ik graag bedanken voor hun supervisie tijdens mijn promotieonderzoek. Naast de fijne samenwerking en discussie hebben jullie ervaring en inzichten enorm geholpen mij op klinisch gebied te ontwikkelen. Verder wil ik graag de leden van mijn promotiecommissie bedanken voor het lezen en de beoordeling van mijn thesis.

Dr. Olaf van Tellingen, stagebegeleider en vriendje van mijn directe collega Dorothe, enorm bedankt voor je begeleiding tijdens mijn stage en de technische discussies. Het wilde nog weleens gebeuren dat de LC-MS/MS complexe technische mankementen had en het was enorm fijn dat je deur altijd open stond om nieuwe ideeën voor troubleshooting op te doen.

Dr. Rawi Ramautar en Dr. Rosilene Burgos, erg bedankt voor jullie begeleiding tijdens mijn eerste masterstage. Door deze stage en begeleiding heb ik mijn affiniteit met academisch onderzoek ontdekt en heb ik dankzij jouw contacten Rawi een mooie stage aan het AVL kunnen doen.

Mijn paranimfen, Dorothe en Mirthe, jullie waren naast de hulp tijdens mijn projecten ook steunpilaren. Als ik er even doorheen zat waren jullie er altijd om mijn frustraties te uiten (en ik hoop andersom ook!). Altijd kritisch, en tikkeltje opstandig, hebben jullie zeker bijgedragen aan het een goede afloop van mijn promotieonderzoek. En Mirthe, zonder jouw hulp had ik waarschijnlijk niet deze unieke positie kunnen krijgen, waarvoor mijn dank! De mooie momenten van onze uitjes zal ik blijven herinneren!

Daan (Vessies/onderDaan), mijn kamergenoot. Elke dinsdag was het een feestje om op kantoor te zijn samen. De disco dinsdag beats zullen niet vergeten worden. Kalpana, oftewel Kalpie, met jou was geen gesprek saai! Ik kijk terug op mooie tijden en een bijzondere bruiloft.

Ruben, de codekunstenaar van ons prachtige instituut, wat ben ik blij dat ik jou heb leren kennen tijdens mijn tijd bij het AVL. Als adoptiecollega kwam je in ons midden en al snel werd duidelijk dat we het goed konden vinden. Een gezamenlijke passie voor lunchsnacks, maar ook voor sport. Nou ja, dat laatste lag misschien meer bij jou. Maar vooral bedankt voor alle mooie momenten de afgelopen 5 jaar. Ik weet zeker dat ik aan je zal denken als ik een paar mooie witte sneakers zie.

De routinemedewerkers en in het bijzonder de ST groep en nog bijzonderder Jenny. De bepalingen, die in dit proefschrift zijn beschreven, zijn uiteindelijk bestemd voor routinematig gebruik. Een nauwe samenwerking was dus nodig met de analisten die ze wekelijks inzetten. Het was erg fijn dat de lijnen kort waren en het overleg vaak soepel verliep. Al vind ik het ergens nog steeds moeilijk, ik weet dat het beheer van de bepalingen nu in de vertrouwde handen is van Jenny.

Zonder hulp van studenten geen succes. Bedankt voor jullie inzet, Maik, Manon, Ditte, Serry, Luuk, Kimberley en Simran. Ik heb het als een eer ervaren jullie te mogen begeleiden.

Dr. Tiny Korse, zonder een goede biobank waren de studies in dit boekje niet mogelijk geweest. Erg bedankt voor je hulp met het verzamelen van monsters en de fijne samenwerking in de Novaria studie. Ravi, ook jij bedankt voor de samenwerking in dit project. Het was mooi om via jou zoveel klinische en gynaecologische inzichten te krijgen.

Mijn vrienden Martijn, Thomas, Eric, Roel, Roderik, Kees, Wim, Jasper en in het bijzonder Tim (deelgenoot in het volgen van dit hobbelige pad) wil ik graag bedanken voor de ontzettend mooie tijden tijdens mijn studiejaren en jeugdjaren. Daarnaast hebben jullie een belangrijk deel uitgemaakt van mijn ontwikkeling en in die zin ook een zekere bijdrage geleverd aan dit geheel.

Ook een goede familiale basis heeft bijgedragen aan een succesvolle afronding mijn proefschrift. Pap, mam en Julia, ondanks dat ik het jullie niet altijd makkelijk heb gemaakt, wil ik jullie ontzettend bedanken voor jullie onvoorwaardelijke steun de afgelopen 33 jaar. En toch wel in het bijzonder mam. Hoe jij, soms tegen wil en dank, je mijn hele leven hebt ingezet om het meeste uit mezelf te laten halen is bewonderenswaardig en verdient alle lof. Ik weet zeker dat ik zonder jou dit alles niet had bereikt.

Na 23 jaar kwam daar mijn schoonfamilie bij, die ik sinds kort ook officieel zo mag noemen. Kees, Mieke, Klaas, Car, Teun, Lucas en Faber, jullie hebben mij een werkelijk tweede thuis gegeven. Het is een warm bad elke keer als ik bij jullie te gast ben en ik kijk nu al uit naar het volgende familieweekend!

“Ik heb het nog nooit gedaan. Dus ik denk dat ik het wel kan.” De stoutmoedige woorden van Pippi Langkous echoën na in jou, Marieke. Je doorzettingsvermogen, mentaliteit en ambities zijn hier voorbeelden van en een grote inspiratie geweest voor de inspanningen die tot dit boekje hebben geleid. Verder vind ik het fantastische dat ik je sinds kort, na een prachtige dag en met trots, mijn vrouw mag noemen en dat we samen richting Canada zijn vertrokken. Ik kijk ontzettend uit naar onze toekomst en avonturen samen.

Curriculum Vitae

Lennart van Winden was born on the 10th of December 1989 in Rotterdam. He grew up in Poortugaal and in 2009, he received his high school diploma at the Marnix Gymnasium in Rotterdam. Afterwards, he moved to Leiden to study BioPharmaceutical Sciences (BFW), for which he obtained his Bachelor of Science degree in 2015. In his subsequent Master of Science program Systems Pharmacology of BFW, Lennart spent 9 months at the department of Analytical BioSciences at the Leiden Academic Centre for Drug Research. Here, he investigated oxidative stress metabolomics during the respiratory burst in neutrophil-like cells. He performed a second internship at the department of Laboratory Medicine of the Netherlands Cancer Institute (NKI) under the supervision of Dr. Huub van Rossum and Dr. Olaf van Tellingen developing and validating a serum testosterone liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay for routine clinical application. After receiving his Master of Science degree in 2017, he was invited by Dr. Huub van Rossum to start as a research analyst at the NKI. Under the supervision of Dr. Huub van Rossum, in 2018, Lennart initiated a PhD project aimed at investigating the application of blood-based sex steroid LC-MS/MS assays in prostate and ovarian cancer diagnostics. From October 2022, Lennart continued his academic efforts in cancer research at the lab of Dr. Phedias Diamandis of the University Health Network (Toronto, Canada).