RESEARCH

Discordance between testosterone measurement methods in castrated prostate cancer patients

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Abstract

Failure to suppress testosterone below 0.7 nM in castrated prostate cancer patients is associated with poor clinical outcomes. Testosterone levels in castrated patients are therefore routinely measured. Although mass spectrometry is the gold standard used to measure testosterone, most hospitals use an immunoassay method. In this study, we sought to evaluate the accuracy of an immunoassay method to measure castrate testosterone levels, with mass spectrometry as the reference standard. We retrospectively evaluated a cohort of 435 serum samples retrieved from castrated prostate cancer patients from April to September 2017. No follow-up of clinical outcomes was performed. Serum testosterone levels were measured in the same sample using liquid chromatography coupled with tandem mass spectrometry and electrochemiluminescent immunoassay methods. The mean testosterone levels were significantly higher with immunoassay than with mass spectrometry (0.672 ± 0.359 vs 0.461 ± 0.541 nM; $P < 0.0001$). Half of the samples with testosterone ≥0.7 nM assessed by immunoassay were measured <0.7 nM using mass spectrometry. However, we observed that only 2.95% of the samples with testosterone <0.7 nM measured by immunoassay were quantified ≥0.7 nM using mass spectrometry. The percentage of serum samples experiencing testosterone breakthrough at >0.7 nM was significantly higher with immunoassay (22.1%) than with mass spectrometry (13.1%; $P < 0.0001$). Quantitative measurement of serum testosterone levels >0.7 nM by immunoassay can result in an inaccurately identified castration status. Suboptimal testosterone levels in castrated patients should be confirmed by either mass spectrometry or an immunoassay method validated at low testosterone levels and interpreted with caution before any changes are made to treatment management.

Key Words

- androgen deprivation therapy
- immunoassay
- mass spectrometry
- prostate cancer
- testosterone

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Introduction

Androgen deprivation therapy (ADT) is used to treat recurrent and advanced PCa (1, 2). ADT blocks the testicular production of androgens thus inhibiting PCa cell growth (3). Serum testosterone levels are a prognostic factor widely used to monitor ADT effectiveness. In fact, several studies demonstrated that testosterone

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levels in castrated PCa patients can predict either time to castration or abiraterone resistance and that patients experiencing testosterone breakthroughs have higher rates of biochemical failure (4, 5, 6, 7, 8, 9). Klotz *et al.* recently published a consensus management algorithm for castrated patients. This algorithm involves regular testosterone measurements to guide clinicians in their decision making. Therefore, having accurate testosterone measurements is instrumental in the clinical management of PCa patients under ADT (10).

In clinical practice, testosterone levels are usually measured by either electrochemiluminescent- or radioimmunoassays. Historically, the lower limit of quantification (LLOQ) of these methods was 1.7 nM (50 ng/dL). Therefore castrate testosterone levels were defined as <1.7 nM; despite this, surgical castration achieves levels of testosterone around 0.7 nM (11). Recent studies have suggested that testosterone levels <0.7 nM under ADT are associated rather with a longer time to castration-resistant prostate cancer (CRPC) or death compared to higher testosterone levels (4, 12, 13, 14, 15, 16, 17). Contemporary electrochemiluminescent immunoassay methods have a lower LLOQ (around 0.4 nM), thus lower castration testosterone levels are now targeted (<0.7 nM) (10).

Mass spectrometry (MS) has been shown to be more sensitive and accurate than immunoassay (IA) methods. Although liquid chromatography–tandem MS (LC–MS/MS) is recognized as the gold standard method to measure steroid levels (18), MS requires specialized equipment and highly skilled technicians, which prevents its use in most clinical laboratories. Comparative studies of MS and IA testosterone measurement methods at castrated levels are thus warranted to determine whether improved sensitivity and accuracy could enhance the clinical management of patients receiving ADT.

The objective of this study was to evaluate the accuracy of an IA method to measure low testosterone levels, with a LC–MS/MS method as the reference.

Materials and methods

Study cohort

This study was approved by the CHU de Québec-Université Laval institutional review board (2017-3431). This retrospective study investigated serum testosterone measurements performed between April and September 2017 at the CHU de Québec-Université Laval where all testosterone measurement requests are initially measured by Roche Diagnostics electrochemiluminescent IA (19). At our institution, when serum testosterone levels are measured <3 nM, they are automatically submitted for testosterone measurement using LC–MS/MS adapted from a published method (20) by the clinical laboratory (see below). All testosterone measurements took place at the CHU de Québec-Université Laval, which is, to the best of our knowledge, the only institution in Québec to use both methods to systematically evaluate circulating testosterone <3 nM. Of the 454 patients initially identified, we were able to confirm a PCa diagnosis for 342 patients (Fig. 1). The measurements (n=524) were performed as part of a PCa follow-up. IA method was able to assess testosterone levels above LLOQ (0.416 nM) for 149 serum samples whereas MS detected testosterone levels above LLOQ (0.1 nM) for 435 samples. Table 1 lists the clinical and pathological characteristics of the study cohort.

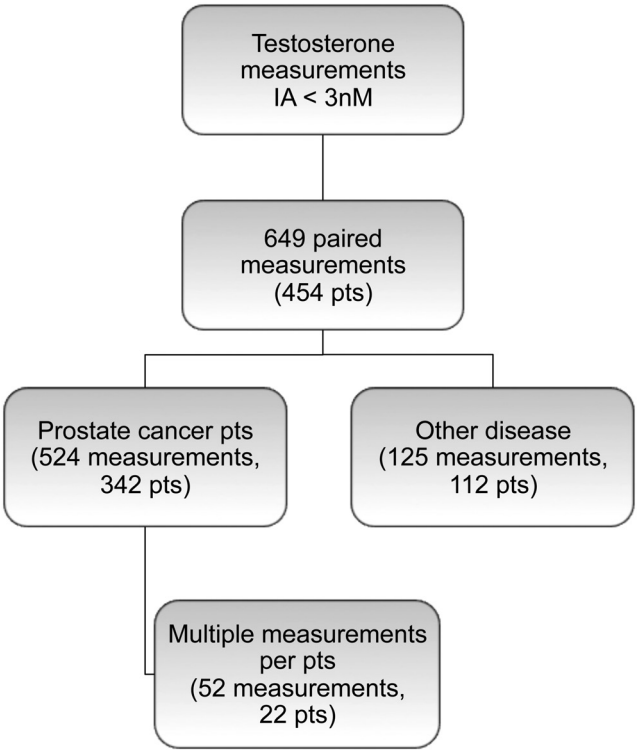

Figure 1
Study scheme for the cohort included in the analysis. Data were extracted from the CHU de Québec-Université Laval biochemistry database for testosterone measurements below 3 nM as measured by an electrochemiluminescent immunoassay (IA) and for which we had corresponding liquid chromatography coupled with tandem mass spectrometry (MS) measurement. The statistical analysis included 435 measurements from 304 patients with histologically confirmed prostate cancer for whom the levels of serum testosterone determined by MS was above the lower limit of quantification (≥0.1 nM). Pts, patients.

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Measurement of serum testosterone by LC–MS/MS

The calibration standards (6PLUS1 Multilevel Serum Calibrator Set MassChrom Steroid Panel 2) and quality controls (QC) (MassCheck Steroid Panel 2 Level I, II and III) were purchased from ChromSystem (Grafelfing, Germany). They are human-based lyophilized material (0–40 nM for calibrators and three levels for QC). A 100 µg/mL solution of testosterone $^{13}C_3$ (Cambridge Isotope Laboratories, Tewksbury, MA, USA) was diluted with LC–MS grade methanol (OmniSolv LC–MS, EMD, Mississauga, ON, CA) to prepare the internal standard working solution (6.86 nM).

Table 1 Clinical and pathological characteristics of the study cohort.

Parameter	Value
Number of testosterone measurements	435
Number of patients with PCa	304
Age at sampling (year)	
Mean (range)	74.03 (39–93)
Clinical stage	
cT1	107
cT2	127
cT3	29
cT4	12
NA	29
Biopsy Gleason score	
4	2
5	5
6	38
7	92
8	75
9	76
10	5
NA	11
Metastasis status at sampling	
M0	189
M1	115
Duration of castration (month)	
Mean at sampling (range)	19.75 (1–160)
Castration method	
LHRH antagonist	33
LHRH agonist	215
Surgical	7
Completed blockade	34
Intermittent	6
None (hypogonad)	6
NA	3
Indication for ADT	
Metastatic disease	115
Neo/adjuvant RxTx	103
Biochemical recurrence	62
NA	18

ADT, androgen deprivation therapy; LHRH, luteinizing hormone-releasing hormone; M0, non-metastatic; M1, metastatic; NA, information not available; PCa, prostate cancer; RxTx, radiotherapy.

To perform protein precipitation, 100 µL of the standard, QC or sample were placed into a 1.5 mL polypropylene microcentrifuge tube. Then, 100 µL of 0.1 M zinc sulfate (Thermo Fisher) in water (LC–MS grade, Purelab Ultra, ELGA) were added. This mixture was then vortexed vigorously for 30 s. After vortexing, 250 µL of internal standard was added and the mixture was vortexed for 1 min followed by a 3-min incubation at room temperature. Samples were then centrifuged for 2 min at 15,000 ***g*** at room temperature. The supernatant was transferred to an autosampler vial and directly injected into the acquity ultrahigh pressure liquid chromatography and online solid phase extraction system (Waters, Milford, MA, USA) using partial loop mode. Method validation was performed following CLSI guidelines (C62 and C57) (21, 22). This method is evaluated monthly by an external validation program (UK NEQAS), which provides three samples containing low testosterone level to assess the quality and accuracy of the measurements.

Chromatographic separation of testosterone from other components using the guard column and analytical columns maintained at 55°C was performed as described (20). The eluate was injected from the LC directly into a XEVO TQ MS tandem mass spectrometer (Waters) as described (20). Transitions (for details on ions see (20)) were monitored in multiple reaction monitoring mode, with a dwell time of 0.155 s. LLOQ (<0.1 nM) was defined as the lowest amount of analyte that can be detected with a coefficient of variation (CV) of 20% and a signal-to-noise ratio (peak-to-peak method) of ≥10.

Measurement of serum testosterone by IA

The testosterone was measured by electrochemiluminescent IA using the automated modular platform from Roche Diagnostics (19). The LLOQ was established as 0.416 nmol/L, and according to the manufacturer the intra-assay CV was 4.6% for a concentration of 0.85 nM.

Statistical analysis

Since the LLOQ for the two methods were different (IA<0.416 nM; MS<0.1 nM), we compared multiple methods to handle values below these thresholds and assessed the bias induced in our analysis (Supplementary Figs. 1 and 2, see section on supplementary data given at the end of this article). The apparatus used for IA provides a testosterone value for measures <0.416 nM, which are associated with a much higher CV (>20%). One of the methods we used involved ignoring the values <0.416 nM

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as given by the IA apparatus for which an MS value ≥0.1 nM was available and to use a prediction model instead. We have fitted a linear regression model using a maximum likelihood method to estimate the testosterone values when MS testosterone dosage was between 0.1 and 0.7 nM (23). Because we sought to reduce the bias associated with IA values <0.416 nM, we chose the prediction model to extrapolate these values.

Statistical analyses were performed using Prism version 7 (GraphPad Software Inc.). To compare the testosterone levels determined by both measurement methods, the Wilcoxon matched-pairs signed rank test was performed based on the histogram distribution of the measurements (Supplementary Fig. 3). *P* values of <0.05 were considered significant.

Results

The IA method overestimates serum testosterone levels in castrated PCa patients

The study cohort included 524 serum samples from PCa patients. The circulating testosterone was measured by employing IA and MS on the same blood sample (Fig. 1). Of these, only 149 serum samples (28.4%) recorded a testosterone level higher than the LLOQ of 0.416 nM using the IA method, compared to 435 samples using MS (LLOQ <0.1 nM; 83.0%). Our analysis was performed on the 435 samples for which a testosterone value was available with the MS method and was used for the paired analysis linear regression model to extrapolate IA values below 0.416 nM (see 'Materials and methods' section for details).

For each testosterone measurement, the difference between the IA and the MS testosterone values was assessed. Analysis of the systematic bias between both testosterone measurement methods was performed using the Bland–Altman plot. The IA method overestimated testosterone levels when compared to MS (mean bias ± S.D.; 0.211 ± 0.260 nM; limits of agreement: −0.300 to 0.721) (Fig. 2A). The overall mean circulating testosterone level was 45.8% higher using the IA (0.672 ± 0.359 nM; CI 95%: 0.638–0.706) than by MS (0.461 ± 0.541 nM; CI: 0.410–0.512; $P < 0.0001$) (Fig. 2B).

We then determined the distribution of patients between the following three categories of testosterone levels: <0.7, 0.7–1.7 and >1.7 nM (Fig. 2C). The mean testosterone value of measures below the castration level

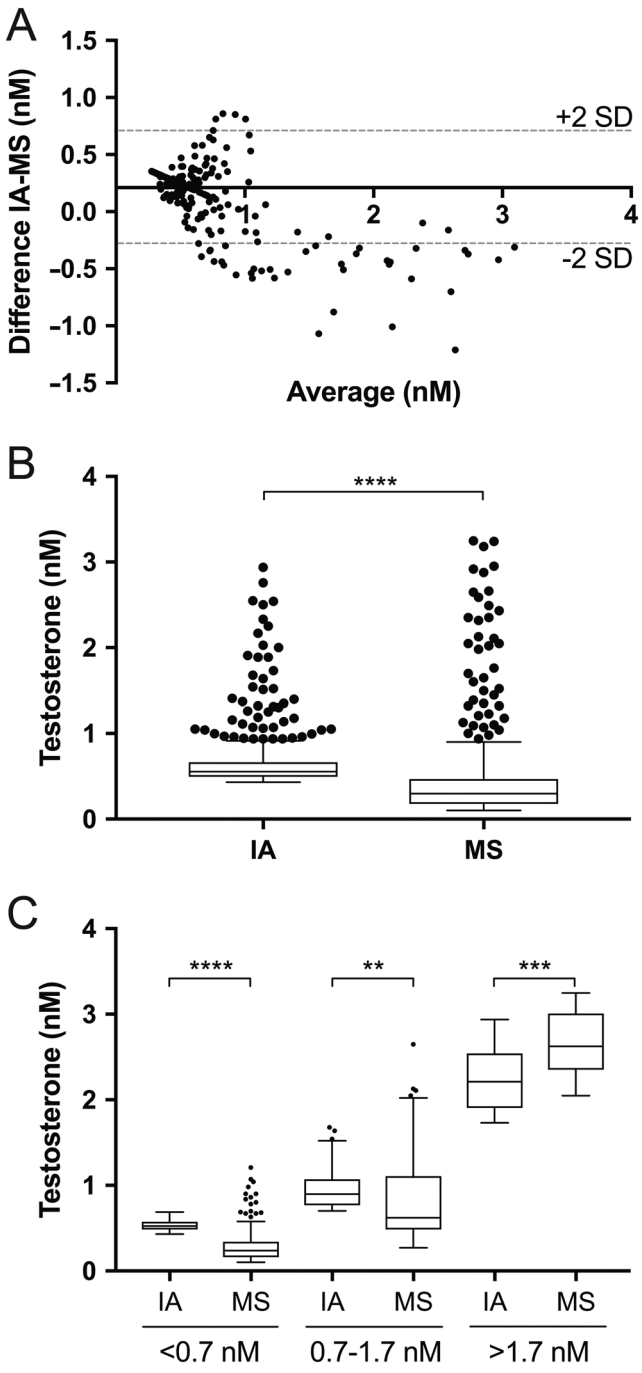

Figure 2
Comparison of electrochemiluminescent immunoassay (IA) and mass spectrometry (MS) to assess low serum testosterone levels reveals discrepancies. (A) Bland–Altman plot of differences between the electrochemiluminescent IA and MS testosterone measurement methods. The *y* axis represents the difference in serum testosterone as determined by the two methods (IA–MS) and the *x* axis represents the mean of the serum testosterone concentrations measured by IA and MS ((MS + IA)/2). The *x* axis intersects the *y* axis at the mean difference between both methods. (B) Mean testosterone level of the overall cohort study; (C) at levels defined for a successful castration. $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

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of <0.7 nM showed that values were higher by 93.5% for this category when IA was used (mean ± S.D.: 0.536 ± 0.061) compared to MS (0.277 ± 0.170; $P<0.0001$). There was also a statistically significant difference between the two methods for values between 0.7–1.7 nM ($P=0.0062$; 14.6% higher for IA than MS) and higher than 1.7 nM ($P=0.0001$; 16.4% lower for IA than MS).

IA serum testosterone measurement often leads to inaccurate classification of the castration status of PCa patients

Using the IA method, 339 samples were <0.7, 82 samples between 0.7 and 1.7, and 14 samples >1.7 nM. Using the MS method, 376 samples were <0.7, 38 samples between 0.7 and 1.7, and 21 samples >1.7 nM. Among the 339 serum samples with a testosterone level <0.7 nM as measured by IA, the MS testosterone measurement was ≥0.7 nM in 10 samples (Fig. 3A). Therefore, IA underestimated the testosterone level in only 2.95% of these samples (mean difference of 38.55%; $P=0.002$) (Fig. 3C). In contrast, 48 out of the 96 samples (50%) with a testosterone measurement ≥0.7 nM by IA were measured <0.7 nM by MS (Fig. 3B). In these samples, IA overestimated testosterone levels by a mean of 77.22% ($P<0.0001$) (Fig. 3D). Therefore, half of the PCa patients who were considered not adequately castrated based on IA were in fact fully suppressed as determined by the gold standard MS measurement of testosterone. In addition, the number of testosterone breakthroughs >0.7 nM was higher with IA (96/435 samples; 22.1%) than with MS (57/435 samples; 13.1%).

MS and IA methods display similar reproducibility over time

For 22 patients undergoing continuous ADT, at least two serum samples (total $n=52$) were analyzed over the 3-month period of the study. The levels of testosterone for these samples, as determined using IA and MS, was higher than the respective LLOQ values for both measurement methods. We used the repeated samples for each patient to compare the reproducibility of both testosterone measurement methods (Fig. 4). The mean variation in the percentage of testosterone levels determined by IA (30.95 ± 24.73%) and MS methods (22.56 ± 22.22%) were not significantly different ($P=0.1004$), suggesting that the reproducibility of both methods in these conditions is similar.

Discussion

In this study including more than 400 testosterone measurements, we demonstrate that the IA method overestimated testosterone levels in a large proportion of patients (Fig. 2A and B). While only 2.95% of the samples with testosterone <0.7 nM measured by IA had in fact testosterone ≥0.7 nM when assessed by the reference MS method, 50% of the samples measured with testosterone ≥0.7 nM by IA had fully suppressed testosterone levels (<0.7 nM) when measured by MS (Fig. 3). This translates into an absolute overestimation of testosterone levels >0.7 nM in 11.0% of the samples (48/435 samples). This finding is important from both a laboratory methodological standpoint and a clinical

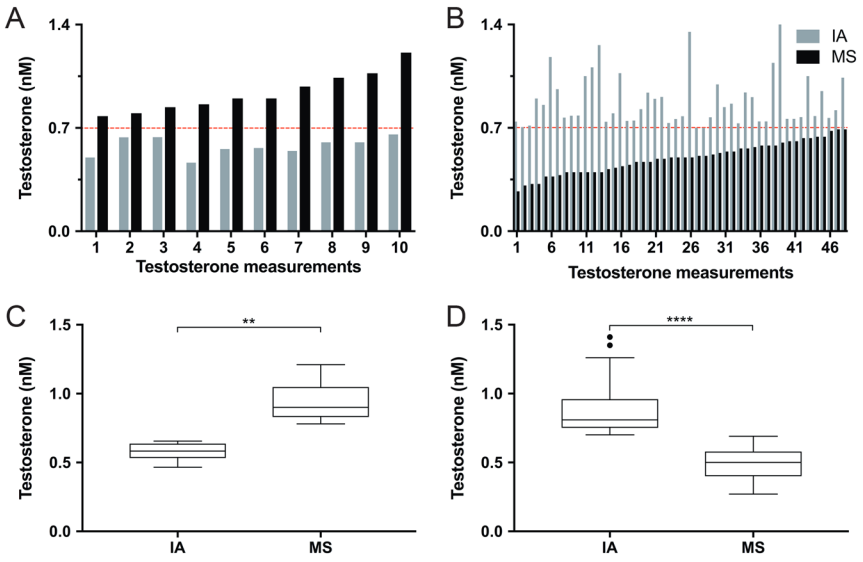

Figure 3
Assessment of castration status by immunoassay (IA) leads to misclassification of prostate cancer patients undergoing androgen deprivation therapy. (A) 2.95% (10 out of 339) of the samples with testosterone <0.7 nM measured by IA were measured as ≥0.7 nM by mass spectrometry (MS). (B) 50% (48 out of 96) of the samples with testosterone ≥0.7 nM measured by IA were measured as <0.7 nM by MS. (C) IA underestimates (in the subgroup described in A) testosterone level <0.7 nM compared to MS by a mean of 38.55%. (D) IA overestimates (in the subgroup described in B) testosterone level ≥0.7 nM compared to MS by a mean of 77.22%. $^{**}P<0.01$; $^{****}P<0.0001$.

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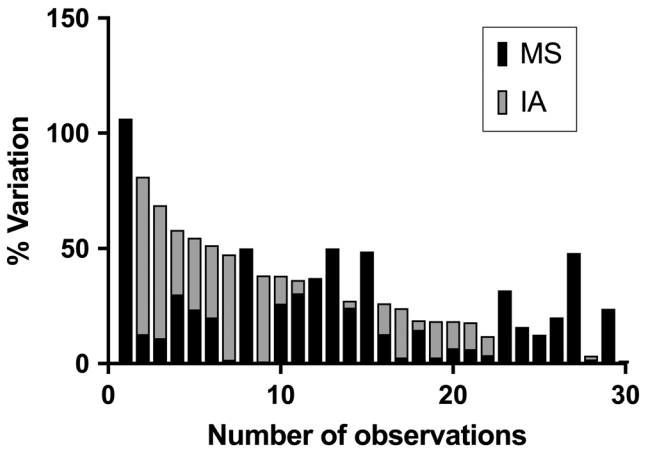

Figure 4
Reproducibility of IA and MS methods assessed using repeated samplings at several timepoints shows no significant difference between the two methods. Serum testosterone level measurements above the lower limit of quantification for both methods were analyzed. Patients receiving intermittent androgen deprivation therapy were excluded from this analysis. The percentage of variation represents the differences in serum testosterone level between each patient's sample for each measurement method ($(IA^{x+1} - IA^{x})/IA^{x}$) or ($(MS^{x+1} - MS^{x})/MS^{x}$).

perspective. Misclassification of a patient's castration status by IA could mislead treatment decisions for PCa patients (Fig. 5). The most important consequence would be to not recognize a patient as being CRPC rather than suboptimally castrated. Based on IA testosterone level determination, prostate-specific antigen (PSA) levels and metastatic status, it is now recommended to either change the ADT drug, or add bicalutamide, enzalutamide or abiraterone acetate (Canadian guidelines) (10, 24). Herein, we propose an updated clinical decision algorithm based on the one by Klotz *et al.* (10), which introduces serum testosterone level measurement by MS when PCa patients under ADT are measured with testosterone levels >0.7 nM by IA (Fig. 5, orange panel). Our study supports a role for MS in the management of advanced PCa under ADT.

Our findings are relevant to previous studies that have demonstrated that testosterone levels <0.7 nM correlate with a longer time to CRPC progression (4, 5). Patients with nadir testosterone levels during the first year of ADT ≤0.7 nM had a longer time to CRPC compared to those with levels either between 0.7 and 1.7 nM or >1.7 nM (10.0 vs 7.21 vs 3.62 years respectively; $P=0.015$) (14, 24). In addition, testosterone breakthroughs above 1.1 and 1.7 nM have been found to be predictors of progression to CRPC when compared to patients without breakthrough (88 vs 137 months; $P<0.03$) (5, 6, 7, 8). Indeed, if half of the patients with testosterone levels >0.7 nM were in fact castrated, the impact of testosterone breakthroughs could be even higher than reported. This is because many patients in the >0.7 nM testosterone group based on IA were likely fully suppressed, contaminating the survival results. In future studies, it will be essential to use an accurate and specific testosterone measurement method at low testosterone levels, such as MS, to clearly identify PCa patients at high risk of progression to CRPC.

It is noteworthy that our results are also in line with those of Morote *et al.* who recently compared the accuracy of another electrochemiluminescent IA (Advia Centaur XPi, Siemens) and LC–MS/MS methods to measure serum testosterone at castrated levels in a smaller cohort of 126 patients (25). Their findings support the observation that IA tends to overestimate testosterone levels compared to MS (mean testosterone level: MS=14.0 ng/dL vs IA=31.9 ng/dL; $P<0.01$) and that, based on IA results, the castration status was wrongly assessed for almost 15% of cases.

There are multiple well-known explanations as to the higher testosterone levels detected by IA relative to MS (26, 27, 28). Other steroids with a close chemical structure to testosterone (i.e. androstenedione, dihydrotestosterone, androstenediol and likely others) may interfere with IA and contribute to the higher readout by this method that lacks specificity (26). Furthermore, adrenal steroids (androstenedione or androstenediol) are not decreased by castration and these, despite having negligible effects on IA at eugonadal testosterone levels (26), could be responsible for higher testosterone levels when measured by IA at castrate levels. This would explain the different results reported between studies performed using castrate and eugonadal testosterone levels (25, 29, 30).

Because all serum testosterone measurements were carried out at the same institution and both measurements performed on the same serum sample, intrapatient and interassay methodological variability were limited. Indeed, we were able to determine the intrapatient measurement variability on repeated measures, which on average was only 31 and 23% for IA and MS, respectively, and not significantly different between methods. Our data thus show that intrapatient intermeasurement variability is about 25%, which is much less than the overestimation of testosterone levels by IA when testosterone is >0.7 nM. These results suggest that with a testosterone level measurement by IA >0.7 nM, confirmation by either MS or an IA method validated at low testosterone levels is more important than repeating the sampling later as suggested previously (15).

Our study does present some limitations. The cohort was only followed over a 3-month period and lacks correlation with the clinical outcomes. However, we believe

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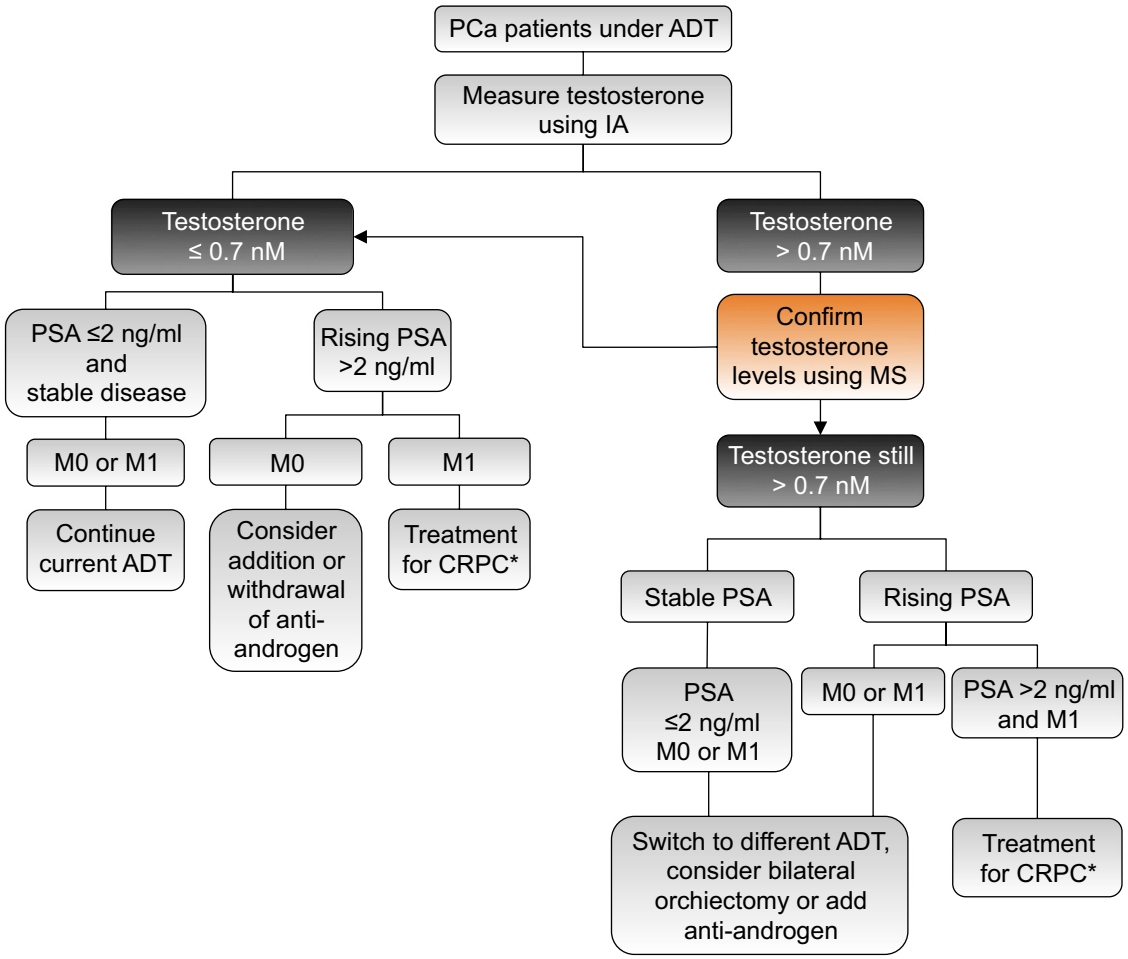

Figure 5
Proposed management algorithm for castrated PCa patients based on this study findings (modified from Klotz *et al.* (10)). The majority of clinical laboratories use immunoassay (IA) methods to measure serum testosterone levels in the clinical follow-up of castrated prostate cancer patients. Currently, for these patients, a testosterone level >0.7 nM entails significant modifications to their treatment course depending on their PSA level and metastatic status. Based on our results, we propose (in orange) an updated algorithm (10) suggesting that for castrated PCa patients with a serum testosterone >0.7 nM measured by IA, confirmation by a method validated at low testosterone levels, such as mass spectrometry (MS), is performed before making any changes to treatment management. *Follow CUA-CUOG guidelines (31). ADT, androgen deprivation therapy; CRPC, castration-resistant prostate cancer; M0, non-metastatic; M1, metastatic; PCa, prostate cancer; PSA, prostate-specific antigen.

that clinical correlation of testosterone measurement was not necessary because our conclusions are strictly limited to analytic method. We have also analyzed all samples, regardless of the treatment received for castration. There was no significant difference in the levels of testosterone between the various forms of castration for IA or MS measurements (Supplementary Fig. 4). Finally, we cannot exclude that other IA could perform better than the one tested.

Conclusions

A significant overestimation of serum testosterone levels near the castration threshold of 0.7 nM was observed in a cohort of castrated PCa patients assessed by IA. The castration status was incorrectly assessed in 13% of patients, leading to inaccurate clinical decisions. A method validated for low testosterone levels like MS should be used as a confirmatory method when serum testosterone levels are >0.7 nM in PCa patients undergoing ADT before changing the clinical management.

Supplementary data
This is linked to the online version of the paper at https://doi.org/10.1530/EC-18-0476.

Declaration of interest
Frédéric Pouliot is or has been a speaker for Astellas, Amgen, Bayer, Genzyme and Sanofi, and a consultant for Amgen, Pfizer, Astellas,

Janssen, Bayer, Genzyme and Sanofi. He holds research agreements with Sanofi and Astellas. Paul Toren holds research agreements with Innocrin Pharma and Roche, and is or has been a consultant for Sanofi Canada, Ferring, Astellas and Abbvie. Laurence Klotz received research support from Ferring, is or has been member of the advisory board for Sanofi, Genzyme and Ferring, and received honoraria from TerSera. Fred Saad is a consultant for Amgen, Abbvie, Astellas, Janssen and Sanofi. His institution has received research grants from AstraZeneca, Astellas, Janssen, BMS, Pfizer, Sanofi and Bayer. Louis Lacombe holds research agreements with Astellas, BMS, AstraZeneca, Roche, Merck, Janssen, Pfizer, Sanofi, Bayer, Tokai, Progenics, MedImmune and Myovant. He is an advisory board member and/or received honoraria for speaking at conferences from Sanofi, Abbott, Astellas, Roche, Janssen, Pfizer and BMS. Yves Fradet is an advisory board member for Bayer, Astellas Pharma, Roche Canada, AstraZeneca/MedImmune, Sanofi Canada and Merck, and has received research funding from Astellas Pharma. The other authors have nothing to disclose.

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Author contribution statement

Study concept and design: Guérette, Pouliot. Acquisition of data: Lemire, Déry, Thériault, Dubois, Guérette. Analysis and interpretation of data: Rouleau, Lemire, Guérette, Pouliot. Drafting of the manuscript: Rouleau, Guérette, Pouliot. Critical revision of the manuscript for important intellectual content: Rouleau, Lemire, Déry, Thériault, Dubois, Fradet, Toren, Guillemette, Lacombe, Klotz, Saad, Guérette, Pouliot. Statistical analysis: Rouleau, Lemire. Obtaining funding: Pouliot. Administrative, technical or material support: None. Supervision: Guérette, Pouliot. Other (specify): None.

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