BJC British Journal of Cancer

www.nature.com/bjc

ARTICLE

Molecular Diagnostics

Urinary oestrogen steroidome as an indicator of the risk of localised prostate cancer progression

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BACKGROUND: Prostate cancer (PCa) is the most common cancer in North American men. Beyond the established contribution of androgens to disease progression, growing evidence suggest that oestrogen-related pathways might also be of clinical importance. The aim of this study was to explore the association of urinary oestrogen levels with clinical outcomes.

METHODS: Urine samples from the prospective multi-institutional PROCURE cohort were collected before RP for discovery ($n = 259$) and validation ($n = 253$). Urinary total oestrogens (unconjugated + conjugated), including oestrone and oestradiol, their bioactive and inactive catechol and methyl derivatives ($n = 15$), were measured using mass spectrometry (MS).

RESULTS: The median follow-up time for the discovery and replication cohorts was 7.6 and 6.5 years, respectively. Highly significant correlations between urinary oestrogens were observed; however, correlations with circulating oestrogens were modest. Our findings indicate that higher levels of urinary oestriol and 16-ketoestradiol were associated with lower risk of BCR. In contrast, higher levels of 2-methoxyestrone were associated with an increased risk of development of metastasis/deaths.

CONCLUSIONS: Our data suggest that urinary levels of oestriol and 16-ketoestradiol metabolites are associated with a more favourable outcome, whereas those of 2-methoxyestrone are associated with an elevated risk of metastasis after RP. Further studies are required to better understand the impact of oestrogens on disease biology and as easily accessible urine-based risk-stratification markers.

British Journal of Cancer https://doi.org/10.1038/s41416-021-01376-z

BACKGROUND

Prostate cancer (PCa) remains the most frequent cancer in North American men[1] and the second most common cancer in men worldwide with over 1.2 million incident cases diagnosed in 2018 and over 350,000 deaths.[2] A recent study showed that the global burden of PCa is steadily increasing worldwide as well as the global incidence of the disease.[3] It remains however a clinical challenge to predict the evolution of localised PCa at diagnosis. Clinical and pathological parameters bring important prognostic information, and scores like the CAPRA and CAPRA-S, were developed to predict the risk of BCR after radical prostatectomy (RP) in patients with localised disease.[4] Despite this clinical and pathological information, additional patients' related factors are needed to better understand disease progression at early stages and to help anticipate the evolution of this heterogeneous disease.[5] Knowing that ~25–30% of patients develop clinically evident recurrence in 15 years post RP,[6–8] the incorporation of additional and non-invasive prognostic markers identifying these at-risk patients could further help clinicians and patients select on the best treatment and follow-up approaches to individualise care.

Most of the research on sex steroids was focused on androgens, such as testosterone (T) and dihydrotestosterone (DHT), and on the androgen-receptor (AR) axis, which are known to play a major role in PCa pathophysiology, evolution and treatment strategies.[9–14] However, growing evidence suggested that oestrogens might assist androgens in perpetuating carcinogenesis.[15–21] Indeed, oestrogens are closely interrelated with androgens, notably due to the fact that T is the precursor of oestradiol (E_2). In animal models, when E_2 is combined with T, PCa incidence rises from 35 to 40% with androgen alone compared to 90–100% with E_2.[15] Moreover, *CYP19A1*-(aromatase) knockout mice, which lack oestrogen production, have elevated circulating T levels but they do not develop cancer.[18] These observations are consistent with the fact that both types of hormones might be required for prostate carcinogenesis.[16,19,20] In addition, the aromatase gene expression is altered in PCa,[22,23] and polymorphisms in the *CYP19A1* and *ESR1* genes were both associated with progression in localised and advanced CRPC disease under androgen-deprivation therapy.[24,25] More recently, aromatase expression in PCa cancer cells was associated with the development of metastasis through oestrogen-responsive elements in the *MMP-12* gene.[26] Besides, the potential role of downstream oestrogenic biotransformation pathways is supported by the demonstration of a link between oestrogen-metabolising genes such as *CYP1B1* and catechol-O-methyltransferase (*COMT*) and the risk of PCa.[27] Indeed,

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Received: 18 January 2021 Revised: 8 March 2021 Accepted: 16 March 2021
Published online: 07 April 2021

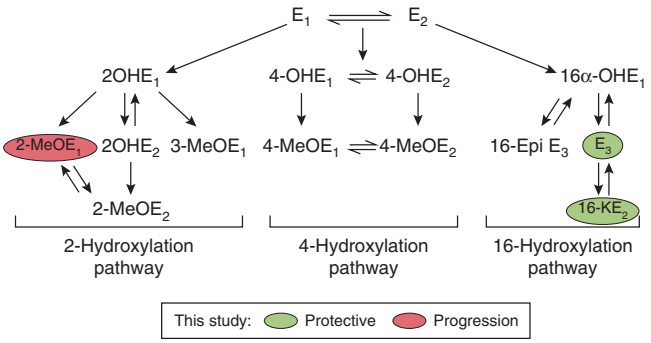

Fig. 1 Simplified biotransformation pathways for parent oestrogens, their bioactive and inactive catechol and methyl derivatives ($n = 15$) measured in this study. The associations of increasing levels of urinary oestrogens and clinical outcome observed in this study are highlighted in green (protective) and red (progression). E_1: oestrone, E_2: oestradiol, 2-OHE_1: 2-hydroxyestrone, 2-OHE_2: 2-hydroxyestradiol, 4-OHE_1: 4-hydroxyestrone, 4-OHE_2: 4-hydroxyestradiol, 16α-OHE_1: 16α-hydroxyestrone, E_3: oestriol, 16-KE_2: 16-ketoestradiol, 16-epiE_3: 16-epiestriol, 2-$MeOE_1$: 2-methoxyestrone, 2-$MeOE_2$: 2-methoxyestradiol, 3-$MeOE_1$: 2-hydroxy-3-methoxyestrone, 4-$MeOE_1$: 4-methoxyestrone, 4-$MeOE_2$: 4-methoxyestradiol.

hydroxylation of oestrone (E_1) and E_2 by CYP enzymes can generate bioactive 2-hydroxy-catechol oestrogens (2-OHCE), 4-OHCE and 16-OHCE (Fig. 1). Unlike 2-OHCE, 4-OHCE and 16-OHCE retain oestrogenic activity and oestrogen-receptor (ER) binding affinities.[28–31] Further, oxidation of CE derivatives to quinones can be deactivated by reduction with the quinone reductases' (NQO1 and NQO2) detoxification pathway or by covalently binding to DNA.[32,33] The depurination of DNA adducts formed from 4-OHCE can generate mutations, whereas in contrast, 2-OHCE lacks carcinogenic potential.[34–41] As a subsequent step, COMT can add a methyl group to CEs and the *O*-methylation process of 2-OHCE to 2-methoxy-CE (2-MeOCE) yields a metabolite that was shown to potently inhibit cell proliferation, tubulin activity and angiogenesis.[28,29,42–45] In addition, intraperitoneal infusion of 4-OHE_2 in animal models shows that the prostate region susceptible to neoplastic transformation has less protection by COMT and NQO detoxification pathways.[41]

Based on these findings, the purpose of this study was to evaluate whether parent oestrogen levels and their bioactive and inactive metabolites measured in urine of cancer patients are associated with adverse prognostic factors, biochemical recurrence (BCR) and development of metastasis/deaths in patients with localised PCa treated by prostatectomy. We used a mass spectrometry (MS) method with high sensitivity to measure levels of fifteen urinary oestrogens, including parent oestrogens, CEs and MeOCE to study a multi-institutional prospective PROCURE cohort[46] divided into discovery ($n = 259$) and replication/validation ($n = 253$) cohorts.

METHODS

Data from the prospective PROCURE PCa cohort

The multi-institutional prospective PROCURE biobank was previously described.[47–49] Briefly, the overall cohort includes 2018 cases recruited between 2007 and 2012 at four university hospital centres in the Province of Québec in Canada (Montreal, McGill, Québec and Sherbrooke). All selected patients for this study had localised PCa at the time of diagnosis, underwent RP and did not receive androgen-deprivation therapy prior to urine sampling. For the discovery cohort, 259 patients were randomly selected in low- ($n = 51$), intermediate- ($n = 110$) and high-risk ($n = 98$) categories, providing an ~2:1 ratio with low-risk disease, to test their links with aggressive disease and adverse clinical outcomes such as BCR and metastasis. A predefined sample-size selection composed of a higher percentage of high-risk patients was retained to detect a hazard ratio of 0.60 for BCR with a power of at least 80% at a 0.05 significance level with a standard deviation varying from 5 to 50. For the validation of positive findings, a cohort of 253 patients was also randomly selected from the same PROCURE cohort. Descriptive clinical and pathological characteristics of the patients studied from the discovery and validation PROCURE cohorts are shown in Table 1. The median follow-up time was 7.6 years for the discovery cohort and 6.5 years for the validation cohort. Serial prostate-specific antigen (PSA) measurements and clinical data were gathered during follow-up. All participants provided written informed consent and the CHU de Quebec research ethics committee approved the research protocol (#2012-362). After prostatectomy, patients were seen regularly and PSA was measured every 3 months for two years, every 4–6 months for 2 years and then every 6–12 months or at the discretion of the physicians. BCR was defined as (1) the occurrence of a confirmed PSA > 0.2 ng/mL any time after surgery or (2) a detectable PSA of <0.2 ng/mL that triggered the initiation of salvage radiation or androgen-ablation therapy, as previously described.[46]

Urinary oestrogen levels measured by mass spectrometry (MS) coupled to liquid chromatography

Urine samples were collected at a preoperative visit and centrifuged at 600×*g* for 10 min at 4 °C using standardised procedures and banked at −80 °C until analysis.[47] Plasma levels of steroids, including T, E_2 and E_1, were available from a previous study of this cohort.[46] Fifteen oestrogens and their metabolites illustrated in Fig. 1 were extracted and quantified in urine samples using MS coupled to liquid chromatography, as previously described[50] and using a liquid chromatographic separation described by Franke et al.[51]. The approach utilises hydrolysis procedures to release the glucuronide and sulfate conjugates of each oestrogen and therefore represents the sum of unconjugated and conjugated quantified hormones.[50] The measured steroids in 250 µL of urine were as follows: oestrone (E_1), oestradiol (E_2), oestriol (E_3), 16-epiestriol (16-epiE_3), 16-ketoestradiol (16-KE_2), 16α-hydroxyestrone (16α-OHE_1), 2-methoxyestrone (2-$MeOE_1$), 2-methoxyestradiol (2-$MeOE_2$), 2-hydroxyestrone (2-OHE_1), 2-hydroxyestradiol (2-OHE_2), 2-hydroxy-3-methoxyestrone (3-$MeOE_1$), 4-methoxyestrone (4-$MeOE_1$), 4-methoxyestradiol (4-$MeOE_2$), 4-hydroxyestrone (4-OHE_1) and 4-hydroxyestradiol (4-OHE_2). Reference steroids were purchased from Steraloids (Newport, RI, USA). Internal standards (deuterated steroids) were purchased from C/D/N Isotopes, Inc. (Pointe Claire, Québec, Canada) and were added to samples. Three low and high hormone-concentration quality-control replicates were included in each run, and all metabolite coefficients of variation were <10%. The limit of quantification, defined as the minimum value at which the ratio of signal-to-noise was ≥5:1, for all these oestrogens, was 10 pg/mL in this biological fluid. Values of catechol oestrogens observed below the lower limit of quantification (LLOQ) were considered as undetected, even if detected above the limit of detection (LOD). All metabolites were detected in all patients, except 4-$MeOE_2$, 4-$MeOE_1$ and 4-OHE_2 that were above the LLOQ in 21%, 74% and 47% of the discovery cohort, respectively.

Urinary creatinine measurements

Normalisation of urinary oestrogen levels was achieved through urinary creatinine levels, measured by MS coupled to liquid chromatography, and reported as pg of steroid per mg of creatinine, in order to standardise the results and ensure comparability with previous studies.[29] Briefly, 20 µL of urine (standard, QC or sample) were mixed with 5 mL of water and 100 µL of internal standard (creatinine d3). Then 10 µL of this dilution were mixed with 300 µl of H_2O and injected into the MS. The chromatographic separation of creatinine was achieved using an Agilent 1200 (Agilent Technologies, Ville St-Laurent, QC, Canada). Separation was performed with an ACE Phenyl column

Table 1. Descriptive characteristics of studied patients from the PROCURE cohort.

Characteristics	Discovery cohort, $n = 259$	%	Validation cohort, $n = 253$	%
Mean age at diagnosis (yr)	62.2		63.1	
SD	6.1		6.3	
Range	45.4–78.7		47.0–76.5	
Median follow-up time (years)	7.6		6.5	
Biochemical recurrence (BCR)	127	49.0	139	54.9
Development of metastasis	19	7.4	24	9.5
Deaths	29	11.3	20	7.9
PSA at diagnosis				
≤10	212	81.9	200	79.1
>10 to 20	33	12.7	39	15.4
>20	14	5.4	14	5.5
Pathologic Gleason score				
≤6	61	23.6	54	21.3
7				
3 + 4	71	27.4	77	30.4
4 + 3	42	16.2	63	24.9
≥8	85	32.8	59	23.3
Pathological T stage				
≤pT2c	142	54.8	122	48.2
pT3a	81	31.3	68	26.9
≥pT3b	36	13.9	63	24.9
Nodal invasion				
pN_0 or pN_x	242	93.4	230	90.1
pN_1	17	6.6	23	9.1
Margin status				
Positive	114	44.0	132	52.2
Negative	145	56.0	121	47.8

(100 × 4.6-mm ID, particle size 3.0 µm, Canadian Life Science, Peterborough, Canada). A binary mobile phase, consisting of H_2O with 1 mM ammonium formate and 0.1% formic acid (solvent A), and MeOH with 1 mM ammonium formate and 0.1% formic acid (solvent B) was used at a flow rate of 0.9 mL/min. Analytes were eluted using the following program: 0–1 min, isocratic 10% B; 1–1.5 min, linear gradient 10–12.5% B; 1.5–1.6 min, linear gradient 12.5–90% B; 1.6–2.6 min, isocratic 90% B; 2.6–2.7 min, linear gradient 90–10% B; 2.7–6.0 min, isocratic 10% B. The API 3200 triple-quadrupole MS instrument (AB Sciex, Concord, ON, Canada) was equipped with a turbo ion-spray source, configured in multiple reaction-monitoring (MRM) mode. MRM transitions were 114.3→44.1 for creatinine and 117.2→47.2 for creatinine d3. Electrospray ionisation was performed in the positive mode with an ionisation voltage of 5000 V, a declustering potential of 36 V, collision energy of 27 V and a heater probe temperature at 500 °C. Analyst software version 1.6.2 (AB Sciex, Concord, ON, Canada) was used for system control and data analysis.

Statistical analysis

Quantitative variables were described as mean, standard deviation (SD), range, median and interquartile range (Q1;Q3), and qualitative variables as frequencies and percentages. Spearman correlations (r_s) between urinary oestrogens were obtained and tested for H0: $r_s = 0$. The associations between clinical prognostic parameters (PSA, Gleason score, pT) and natural logarithm-transformed urinary oestrogen levels were estimated using generalised linear regression modelling adjusted for age and body mass index (BMI). The log transformation of urinary oestrogens was used due to the non-normal distribution of the variables as performed in studies similar in scope.[52,53] A weighted multivariable proportional hazard Cox model adjusted for age, surgical margin, pN, PSA (continuous variable), pT (<pT3a, pT3a, and >pT3a) and Gleason score (GS 6, GS3 + 4, GS4 + 3 and GS > 7) was used to evaluate the risk of BCR and metastasis/deaths according to urinary oestrogen levels. BMI was included in the final multivariable model as a continuous variable. A P value of < 0.05 was considered statistically significant. Based on the fact that (i) all tested hormones are positioned in the same metabolic pathway and (ii) we validated findings in a separate dataset, no correction for multiple testing was performed since no random hypotheses were tested herein. The software used was SAS 9.4 by SAS Institute Inc. (Cary, NC).

RESULTS

Correlations between urinary oestrogens and their metabolites

The parent oestrogen E_1 and its metabolites E_3 and 2-OHE_1 were the most abundant urinary oestrogens found in patients at 4875.6 pg/mg creatinine (95% CI: 4566.3–5184.9), 4852.6 pg/mg creatinine (95% CI: 4470.1–5235.1) and 2810.7 pg/mg creatinine (95% CI: 2573.8–3047.7) (Supplementary Table 1). E_2 was the fourth most abundant oestrogen measured in urine with 1318.5 pg/mg creatinine (95% CI: 1205.1–1431.9). Spearman correlations revealed that urinary parent oestrogens E_1 and E_2 strongly correlated ($r_s = 0.76$, $P < 0.001$) with each other and also with the levels of 2-OHE_1, 2-OHE_2, 16-KE_2 and 16-epiE_3 ($P < 0$ .001) (Fig. 2).

Levels of urinary oestrogens and their correlation to circulating levels of parent oestrogens and testosterone (T)

Moderate correlations were observed between circulating T, E_2 and E_1 and urinary oestrogen levels, with the most significant being with E_2 in urine. For example, urinary E_2 correlated with plasma E_2 with a Spearman correlation value of $r_s = 0.133$ ($P = 0.032$). The strongest correlations were plasma E_2 with 16-KE_2 ($r_s = 0.246$, $P < 0.0001$) and E_3 ($r_s = 0.230$, $P = 0.0002$). Plasma T levels correlated to 16-KE_2 ($r_s = 0.152$, $P = 0.014$). All correlations are presented in Supplementary Table 2.

Relationships between established prognostic factors and urinary oestrogen levels

No significant associations between urinary oestrogens and PSA levels were observed (Supplementary Table 3). In the discovery cohort, 4-$MeOE_1$ and 4-OHE_1 urinary levels were linked to Gleason scores (Supplementary Table 4), but these two associations were not maintained in the validation cohort (not shown). Urinary creatinine levels were not associated with Gleason scores ($P = 0.947$). Urinary oestrogen levels were not associated with pT staging (Supplementary Table 5). Both 2-$MeOE_1$ and 4-OHE_2 were associated with changes in body mass index (BMI) (Supplementary Table 6).

Urinary oestrogen levels and the risk of progression after RP

As shown previously for the entire PROCURE cohort,[49] BMI was not associated with clinical outcome herein (hazard ratio (HR) = 0.98, 95% CI: 0.93–1.05, $P = 0.625$). In multivariable analyses, increasing levels of urinary E_3 were associated with a reduced risk of BCR with HR values of 0.66 (95% CI: 0.43–0.98, $P = 0.038$) in the discovery cohort and 0.74 (95% CI: 0.52–1.04, $P = 0.086$) in the validation cohort. A HR of 0.74 (95% CI: 0.60–0.91, $P = 0.006$) is reported for the combined cohorts. Increased levels of the oestradiol

	E_1	E_2	16α-OHE_1	16-KE_2	16-epiE_3	2-OHE_1	2-OHE_2	4-OHE_1	4-OHE_2	2-$MeOE_1$	2-$MeOE_2$	3-$MeOE_1$	4-$MeOE_1$	4-$MeOE_2$
E_1														
E_2	0.755													
16α-OHE_1	0.217	0.237												
16-KE_2	0.418	0.406	0.675											
16-epiE_3	0.534	0.610	0.497	0.738										
2-OHE_1	0.552	0.450	0.041	0.153	0.242									
2-OHE_2	0.512	0.449	-0.027	0.178	0.348	0.706								
4-OHE_1	0.083	0.089	0.042	-0.029	0.006	0.216	0.156							
4-OHE_2	0.055	0.150	0.011	-0.101	-0.063	0.066	0.027	0.093						
2-$MeOE_1$	0.462	0.394	0.047	-0.007	0.104	0.696	0.402	0.210	0.221					
2-$MeOE_2$	0.385	0.308	-0.056	-0.033	0.102	0.465	0.444	0.161	0.225	0.569				
3-$MeOE_1$	0.340	0.304	-0.030	-0.036	0.081	0.550	0.415	0.341	0.103	0.587	0.601			
4-$MeOE_1$	0.300	0.307	0.157	0.208	0.173	0.319	0.288	0.163	0.219	0.232	0.309	0.371		
4-$MeOE_2$	0.027	-0.017	0.105	0.130	0.018	0.012	0.098	0.096	0.218	-0.053	0.116	0.044	0.324	

> 0.75 ($P < 0.001$)
> 0.5 – 0.75 ($P < 0.001$)
> 0.25 – 0.5 ($P < 0.001$)
> 0.25 ($P < 0.05$)
< 0.25 ($P < 0.001$)
$P > 0.05$

Fig. 2 Correlations between urinary oestrogens in PCa. Spearman correlations between total urinary oestrogen levels measured in PCa patients in the discovery cohort ($n = 259$). The level of correlations between urinary oestrogens is as indicated in colors.

metabolite 16-KE_2 were also associated with a favourable outcome, with a HR of 0.64 for BCR (95% CI: 0.44–0.92, $P = 0.016$) in the discovery cohort, with a HR of 0.75 (95% CI: 0.54–1.04, $P = 0.082$) in the validation cohort and with a HR of 0.75 (95% CI: 0.63–0.90, $P = 0.002$) when the cohorts are combined. HR values for BCR for all tested urinary oestrogens are shown in Figs. 3 and 4. Furthermore, increasing levels of urinary 2-$MeOE_1$ were linked with a greater risk of development of metastasis/deaths during follow-up with HRs of 1.58 (95% CI: 1.05–2.40, $P = 0.028$) in the discovery cohort and 1.54 (95% CI: 1.08–2.21, $P = 0.016$) in the validation cohort, and with a HR of 1.50 (95% CI: 1.21–1.88, $P = 0.0003$) when the cohorts are combined. HR values for the development of metastasis for all tested hormones are shown in Figs. 3 and 4.

DISCUSSION

In this study, we performed a comprehensive evaluation of the association between preoperative urinary oestrogens and their metabolites and the risk of disease aggressiveness and cancer evolution in patients undergoing RP as the sole initial curative procedure. The findings demonstrated no significant associations with pathological factors; however, significant associations were observed between specific urinary oestrogens and clinical outcomes. Our study suggests that higher urinary levels of E_2 metabolites, E_3 and 16-KE_2, are predictive of a lower risk of BCR. In addition, high 2-$MeOE_1$ urinary levels were associated with increased metastatic/death events during follow-up.

The notion that oestrogens have an impact on prostate cancer biology is supported by epidemiologic and genetic association studies, as well as functional investigations using animal models and PCa cell lines.[15,32,54–58] Moreover, findings based on circulating measures of steroids support that an elevation of the E_2/T ratio is associated with aggressive PCa.[59] Recently, high circulating E_1 and E_2 levels in castrated men were found to be associated with shorter time to castration-resistant prostate cancer (CRPC) in a subset of patients from the Canadian-led PR.7 trial in men with localised disease.[56]

However, as observed here, urine oestrogens are likely not surrogates of circulating oestrogens since low coefficients of correlation were observed herein. This lack of correlations between preoperative urinary and circulating oestrogens suggests that oestrogens are, at least in part, metabolised by the prostate and PCa cells. Thus, urinary oestrogens levels have the potential to complement the prognostic information of circulating hormones on the risk of disease progression. However, to our knowledge, very few data are available regarding the impact of the urinary oestrogen metabolome on the risk of developing PCa, and none were found that investigated their role as potential prognostic markers. A case–control study by Muti et al., including 113 cases and 317 controls, suggested that the oestrogen metabolic pathway favouring 2-hydroxylation over 16α-hydroxylation might reduce the risk of PCa.[60] A similar study performed by Barba and colleagues, including 26 cases and 110 controls, also suggested a protective effect of the 2-hydroxylation pathway.[61] In African-American healthy men ($n = 77$) attending prostate cancer screening clinic, urinary levels of 2-OHE_1 were also inversely associated with PSA levels.[62] In our study, no associations were found between urinary levels of 2-OHE_1 with clinical and pathological factors, suggesting that this metabolite may be associated with the risk of developing PCa but not with its aggressiveness. Another case–control study, including 77 PCa patients, 77 healthy controls and 37 biopsy-negative controls, demonstrated that higher urinary 16-KE_2 levels were associated with a trend towards a lower risk of developing PCa in a dose-dependent association.[63] In agreement, high urinary 16-KE_2 elimination was associated with a reduced risk of BCR in this study. Our findings suggest that a more efficient urinary clearance of oestrogens of the 16-hydroxylation pathway, namely E_3 and 16-KE_2, might be beneficial for PCa patients in terms of disease progression after surgery. Moreover, 2-$MeOE_1$ was shown to be associated with metastasis/deaths in this study. Indeed, 2-$MeOE_2$, which is located in the same metabolic pathway, was previously shown to be associated with a protective effect on several types of cancer, notably with an apoptotic activity and with anti-angiogenic and anti-proliferative effects.[64,65] These data suggest that patients that excrete more 2-$MeOE_1$, deemed protective, are at increased risk of experiencing the adverse clinical outcome.

The strengths of this study include (1) patients all treated by RP, thus allowing precise evaluation of associations with pathological findings, (2) standardised specimen collection and storage, (3) a comprehensive analysis of 15 oestrogens and their bioactive and inactive derivatives using a tandem MS method coupled to liquid chromatography with high sensitivity, (4) validation of findings in a separate dataset and (5) significant follow-up to assess the impact on BCR after RP. Our study has the following limitations: (1) a limited number of metastatic events, (2) the assessment of total oestrogen urinary levels that comprise both unconjugated and their

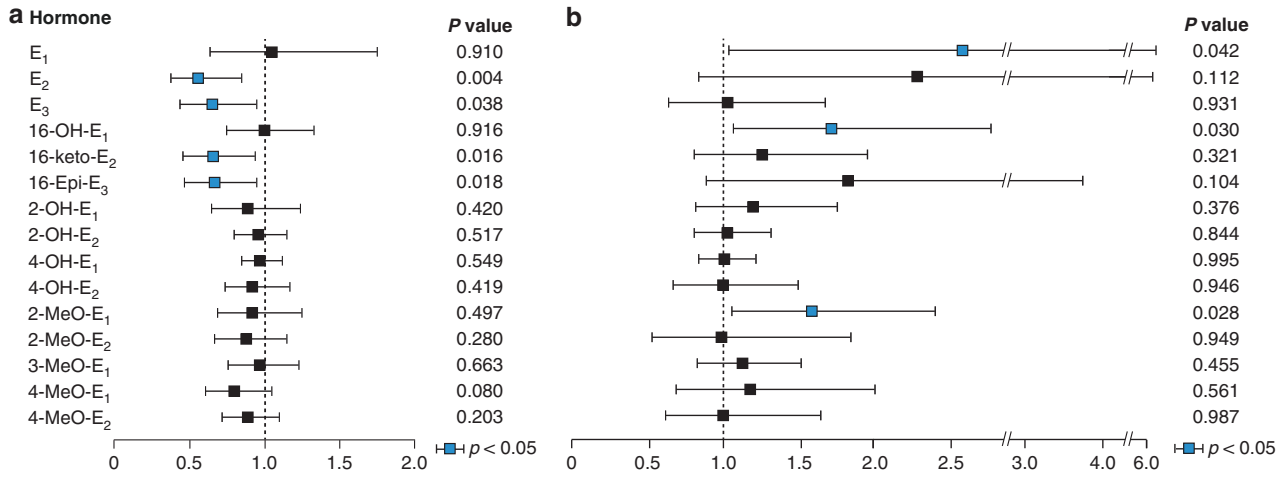

Fig. 3 Urinary oestrogens and the risk of PCa progression. Association between total urinary oestrogens and the risk of (**a**) BCR and (**b**) metastasis/deaths in men with localised PCa in the discovery cohort ($n = 259$). Boxes shown within the main panels represent hazard ratios (HRs) and their 95% confidence intervals in multivariable analyses adjusted for age, PSA, Gleason score, surgical margin, pT, pN and BMI for each hormone. Each unit increment in log-transformed hormone levels is associated with the indicated changes in HR values.

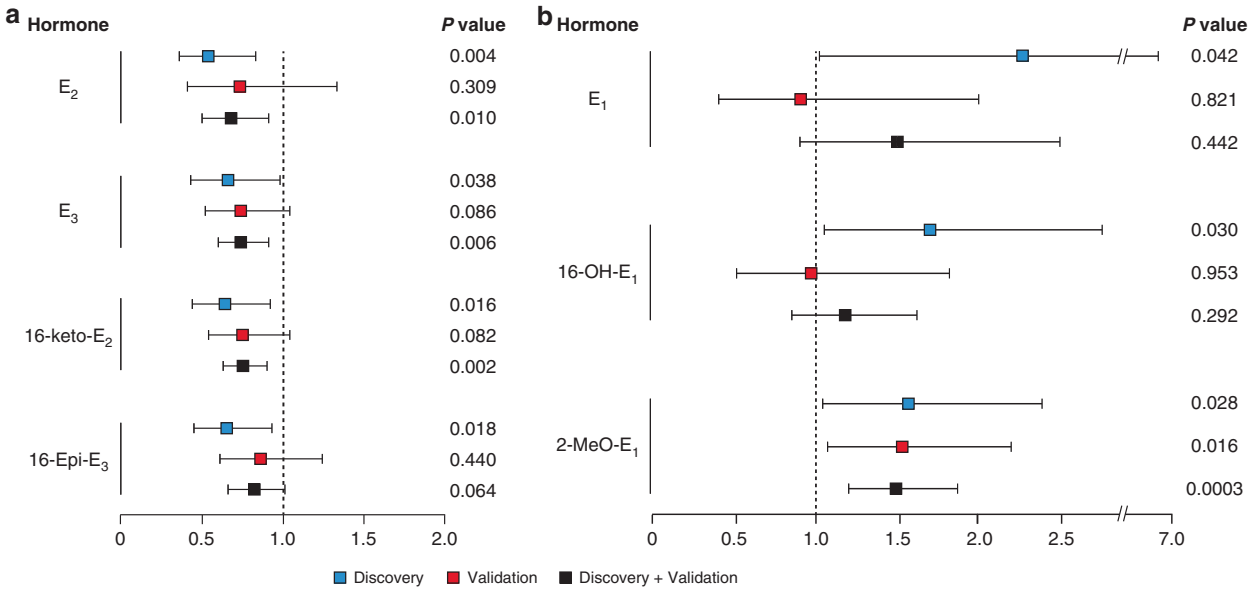

Fig. 4 Validation of urinary oestrogens and the risk of PCa progression. Validation of positive associations between total urinary oestrogens and the risk of (**a**) BCR and (**b**) metastasis/deaths in men with localised PCa in a confirmatory cohort of 253 patients originating from PROCURE. Boxes shown within the main panels represent hazard ratios (HRs) and their 95% confidence intervals in multivariable analyses adjusted for age, PSA, Gleason score, surgical margin, pT, pN and BMI for each hormone. Each unit increment in log-transformed hormone levels is associated with the indicated changes in HR values.

conjugated (sulfate and glucuronide) forms and (3) inaccessibility of intraprostatic oestrogen levels.

We conclude that urinary E_3 and 16-KE_2 levels are associated with less BCR events after RP, and 2-MeOE_1 was associated with disease progression. Our observations are in support of a potential role of oestrogens in progression, but require further evaluation in additional independent cohorts to confirm the associations observed. Our data raise the possibility that an assessment of the oestrogen metabolome in urine samples may provide clinically relevant information that can be used alone or combined with circulating, tumoral and germline markers to better predict clinical outcomes of PCa patients at the time of diagnosis. Additional studies are definitely required to evaluate if urinary oestrogens can be used along with the circulating steroidome to help predict disease evolution at the time of initial diagnosis. Future studies aimed at understanding the impact of the complex oestrogen pathway in PCa are undeniably warranted.

ACKNOWLEDGEMENTS

The authors are thankful to all participating patients and staff at each site associated with the PROCURE Biobank who have made this scientific contribution possible.

AUTHOR CONTRIBUTIONS

Study concept, design and supervision: E.L. and C.G. Steroid measurements: P.C., V.T. and C.G. Statistical analysis: D.S. Interpretation of the data: J.P.E., L.L., P.C., V.T., D.S., A.A., F.S., M.C., S.C., C.G. and E.L. Drafting of the paper: J.P.E., C.G. and E.L. Critical

revision of the paper for important intellectual content: J.P.E., L.L., P.C., V.T., D.S., A.A., F.S., M.C., S.C., C.G. and E.L. Patients' recruitment and clinical data: F.S., L.L., M.C., S.C. and A.A. Obtaining funding: E.L., C.G. and L.L.

ADDITIONAL INFORMATION

Ethics approval and consent to participate All participants provided written informed consent and the CHU de Quebec research ethics committee approved the research protocol, and the study was performed in accordance with the Declaration of Helsinki.

Consent to publish Not applicable.

Data availability The datasets analysed during this study are available from the corresponding author on reasonable request.

Competing interests The authors declare no competing interests.

Funding information This work was supported by research grants from Prostate Cancer Canada (D2013-22 to E.L., C.G. and L.L.), the Cancer Research Society (to C.G.) and the Canada Research Chair Program (to C.G.). E.L. holds a CIHR Clinician-Scientist Award and was the holder of the PCC rising star award (RS2013-59). C.G. holds the Canada Research Chair in Pharmacogenomics (Tier I). The results are based on samples and patient data obtained from the PROCURE Biobank, supported by donations in a partnership with the Cancer Research Society of Canada.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41416-021-01376-z.

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