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TITLE: Estrogen Metabolism and Prostate Cancer Risk: A Prospective Study

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Prostate cancer is the most common cancer among men in the United States (IARC, 1995) and the second most common in the European Community (IARC, 1995). The causes of prostate cancer, however, remain largely unknown, with age, race, and family history being the only established risk factors (Nomura et al., 1997). The prostate gland has historically been considered the prototype of an androgen-dependent organ. However, there is evidence that estrogens may induce mitosis of prostatic epithelial cells in many species, including humans (Leave et al., 1978; Schulze et al., 1987). This report analyzes the association between prostate cancer and estrogen metabolism investigated in a case-control study. In particular, we tested the hypothesis that the pathway favoring 2-hydroxylation over 16α-hydroxylation may be associated with a decrease in prostate cancer risk. This is the final report for the study. During the final year of activity, we completed determinations of the estrogen metabolites using gas-chromatography and carried out statistical analysis. We have also prepared an article for publication of the results (see attached article).
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INTRODUCTION

Prostate cancer is the most common cancer among men in the United States (IARC, 1995) and the second most common in the European Community (IARC, 1995). The causes of prostate cancer, however, remain largely unknown, with age, race, and family history being the only established risk factors (Nomura et al., 1997). The prostate gland has historically been considered the prototype of an androgen-dependent organ. However, there is evidence that estrogens may induce mitosis of prostatic epithelial cells in many species, including humans (Leav et al., 1978; Schulze et al., 1987).

The study analyzes the association between prostate cancer and estrogen metabolism investigated in a case-control study. In particular, we tested the hypothesis that the pathway favoring 2-hydroxylation over 16α-hydroxylation may be associated with a decrease in prostate cancer risk.

This is the final report for the study. During the final year of activity, we completed determinations of the estrogen metabolites using gas-chromatography and carried out statistical analysis. We have also prepared an article for publication of the results (see attached article).
BODY OF REPORT

The study has now been completed with all statistical analysis carried out and a paper detailing the results prepared.

Background

Follow-up of the cohort: The re-call and follow-up of the Western-New York cohort (WNYHC) for the identification of the incident prostate cancer cases and their related control subjects was completed in the previous year of the study. The follow-up was conducted in collaboration with another NIH-funded study, the “Epidemiology of Type-2 Diabetes” study, which is a prospective cohort study based on the same WNYHC cohort (RO1 DK 60587, Dr. R. Donahue, PI, Dr. P. Muti, Co-PI).

The re-call of the cohort started in January 2003. The re-call included participants without history of cancer, cardiovascular diseases, and clinically defined type-2 diabetes at baseline interviewed between 1996-2001. The re-call was also limited to those cohort participants with stored biological samples. Thus, we started the re-call and the follow-up process with a sample of 1,150 cohort participants.

Of the 1,150 cases :-

• 52 were not eligible for medical reasons (too ill with diseases other than those mentioned before),
• 46 had died (for causes other than prostate cancer),
• 22 had moved out of the Erie and Niagara Counties,
• 117 were not contactable by mail or phone.

Overall, we had a sample of 913 re-called participants. Among the 913 participants, we identified :-

• 41 incident prostate cancer cases,
• 232 cases refused to participate in the study (they refer, in the short telephone interview, to having not been diagnosed with prostate cancer),
• 40 were scheduled but then cancelled the appointment,
• 8 were still in-process at the end of the follow-up period (September 30, 2004).

Thus, there were 592 participants available as control subjects.

During the study period, we monitored the occurrence of prostate cancer among the successfully contacted participants. All procedures to re-call, interview and collect biological specimens from the WNYHC Study were similar to the procedures used for baseline recruitment. All eligible participants were initially re-contacted by letter and then by phone (up to twelve callbacks). Participants were invited to attend our recruitment center at the Department of Social and Preventive Medicine, Buffalo, New
York for the clinical examination and to answer questions related to the occurrence of prostate cancer diagnosis between the baseline examination and the re-call time.

**Definition of the matched case-control pairs.**

**Case Identification:** Incident Prostate Cancer Cases: Prostate cancer cases recruited in the study were men who have been diagnosed with incident cytologically and/or histologically confirmed prostate cancer after their recruitment (date at first interview) in the WNYHC Study and before the end of the cohort follow-up period (September, 30, 2004). Prostate cancer cases were identified by their own report at the re-call of the cohort. 32 cases have been validated by their clinical records, while the remaining 9 cases are in the process to be validated. At recruitment, each cohort member signed a consent form giving us permission to request copies of their clinical charts in cases of pathological events related to the WNYHC Study investigations. Through these clinical charts, we are validating the information collected from participants.

**Control Identification:** Eligible controls were all male members of the WNYHC Study who, based on their report, were not diagnosed with prostate cancer at the diagnosis of the related case. For each prostate cancer case, four controls will be randomly chosen after matching for:

a) age (within 3 years);  
b) race;  
c) recruitment date

to control for the effect of long-term preservation of stored urine.

To increase the power of the study (and to reduce the effects of non-diagnosed prostate cancer cases among controls), we used a 1:4 ratio for cases and controls. Therefore, the study hormone determinations were conducted on 41 prostate cancer cases and 164 control subjects.

All hormone determinations by gas-chromatography and data analysis has been completed.

**Hormone Determinations**

For standardization purposes, we collected morning spot urine between 7:00 a.m. and 9:00 a.m. from all participants. We then transferred the aliquoted urine samples to the Eppley Institute, University of Nebraska Medical Center (UNMC), and stored them at -80 °C until analysis. Each analytical sample thawed only once prior to analysis. We handled urine samples identically and located them in the laboratory runs randomly. All laboratory personnel were blinded with regard to case-control status. We analyzed all of the study samples in duplicate. Two-milliliter aliquots of urine underwent partial purification by solid phase extraction (SPE) with a phenyl cartridge (Varian, Palo, Alto, CA) and ultraperformance liquid chromatography/tandem mass spectrometry (LC/MS-MS). We identified the analytes based on their retention time and tandem mass
spectrometry. Standards of the catechol estrogens 2-OHE$_1$(E$_2$) and 16α-OHE$_1$(E$_2$) were purchased from Steraloids Inc. (Newport, RI).

Treatment of urine with glucuronidase/sulfatase led to marginal changes in the levels of estrogen metabolites and in many cases decreased the levels due to incubation for 8 h at 37 °C. To avoid the artifacts and errors introduced by maintaining the urine samples at 37 °C for 8 h, we carried out all the analyses without glucuronidase/sulfatase treatment.

We adjusted urine samples to pH 7 with 1 M NaOH or 1 M HCl. For method development and validation, 2-mL aliquots of charcoal-treated human urine samples were spiked with a total of 250, 500 or 1000 pg of the above compounds (final concentration 0.125, 0.25 and 0.50 pg/µL) and loaded onto the phenyl 100-mg cartridges pre-conditioned with CH$_3$OH and the loading buffer, 10 mM ammonium formate, pH 7. We eluted the compounds of interest from the cartridge after the washing and elution steps. This procedure led to enrichment of the target compounds after elution. We used Charcoal-treated urine (2 mL) in controls. The eluates from both the experimental and control samples were concentrated using a SpeedVac and lypholizer and assessed by LC/MS-MS. To determine the recovery of the standards by SPE method, we compared the corresponding concentrations of experimental and control samples. The recovery of 2-OHE$_1$ and 16α-OHE$_1$ was 97% and 101%.

We carried out the LC/MS analyses throughout a Waters Acquity ultraperformance liquid chromatography (UPLC) system connected with a high performance Quattro Micro triple quadruple mass spectrometer designed for LC/MS-MS operation. We performed the analytical separations on the UPLC system using an Acquity UPLC BEH C18 1.7 µm column (1 X 100 mm) at a flow rate of 0.15 ml/min. The gradient started with 80% A (0.1% formic acid in H$_2$O) and 20% B (0.1% formic acid in CH$_3$CN), changed to 79% A over 4 min, followed by a 6-min linear gradient to 45% A, resulting in a total separation time of 10 min. We then moved the elutions from the UPLC column to the Quattro Micro mass spectrometer.

The ionization method used for MS analysis was ESI in both the positive ion (PI) and negative ion (NI) mode with an ESI-MS capillary voltage of 3.0 kV, an extractor cone voltage of 3 V, and a detector voltage of 650 V. Desolvation gas flow was 600 l/h. Cone gas flow was 60 l/h. Desolvation temperature and source temperature were 200 and 100 °C, respectively. We performed the MS-MS in the multiple reaction monitoring (MRM) mode to produce structural information about the analytes by fragmenting the parent ions inside the mass spectrometer and identifying the resulting daughter/fragment ions. We processed the resulting data and quantified the estrogen metabolites using the QuanLynx software (Waters).

To calculate limits of detection, we injected various concentrations of the analyte to LC/MS-MS, namely 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10, 25, 50 and 100 pg/µl. We considered the injected amount that resulted in a peak with a height at least two or three times higher than the baseline as the limit of detection. The limits of detection of 2-OHE$_1$
and 16α-OHE$_1$ were 18 fmol and 349 fmol, respectively. Intra-assay coefficients of variation for 2-OHE$_1$ and 16α-OHE$_1$ were 3.2% and 3.0%, respectively. Inter-assay coefficients of variation were 1.9% and 3.5%, respectively.

The intra- and inter-individual variability for 2-OHE$_1$, 16α-OHE$_1$ determinations and their ratio expressed as intraclass correlation coefficients (ICC$s$) and lower limit of 95% CI (in parentheses) were 0.70 (0.46), 0.63 (0.35) and 0.78 (0.62), respectively. We have previously provided a detailed description of the procedures related to the reliability assessment.

**Statistical analysis**

We examined distributions for all variables of interest by determining the frequencies, mean, median and measures of variance. To evaluate the statistical significance of the unadjusted associations between case/control status and participants’ characteristics, we used either Fisher’s exact tests or Pearson’s chi-square tests for categorical variables.

We standardized the 2-OHE$_1$ and 16-αOHE$_1$ urinary levels by total urinary creatinine. We used unconditional logistic regression to compute crude and adjusted odds ratios (OR) and 95% confident interval (CI) of Pca in relation to 2-OHE$_1$, 16-αOHE$_1$ and the ratio of 2-OHE$_1$ to 16α-OHE$_1$ by tertiles of urine concentrations. We used the same models to test for significance in trends of associations for any of the independent variables. We computed the cut-off points of the previously mentioned tertiles based on the distributions of estrogen metabolites in control subjects. We analyzed each independent variable separately. Based on the published literature, we identified age, race, education level, BMI and waist-to-hip ratio as possible covariates and tested them using regression models. Although none of them was a confounder for the investigated associations, we included age in years in further analyses based on its biological relevance in prostate carcinogenesis (2).

We verified several sources of potential bias. Because the exclusion of participants with missing data for any of the two outcome variables could have introduced a source of bias in our final sample, we examined data by subsets. Each of the two datasets included those men with no missing data for either urinary levels of 2-OHE$_1$ or 16-αOHE$_1$. We then examined by case-case and control-control comparison the characteristics of the 136 subjects (110 controls and 26 cases) with no data missing for any of the considered variables and those of the subjects (534 controls and 41 cases) who fulfilled our study eligibility criteria. Finally, we compared the subjects in the latter category (575) to the 517 original cohort members who did not join the study because of either not fulfilling the inclusion criteria, being lost to follow-up or not willing to participate.

To date, no data specifically related to any of these three categories exist (i.e. co-morbidity data pertinent to the WNYCS) exist. Thus, we considered these 517 male subjects as part of an overall, although heterogeneous, category. As expected, the 517 males from the original cohort who did not ultimately join our study showed statistically
significant differences compared to the 575 included study participants. We analyzed these data using SPSS version 14.0 (SPSS, Inc., Chicago, IL).

**KEY RESEARCH ACCOMPLISHMENTS**

- Completed bio-assay determinations.
- Report completed.
- Paper with final results submitted.

**REPORTABLE OUTCOMES**

**Results**

We observed a non significant risk reduction in the highest tertile of 2-OHE1 (OR 0.72, 95% CI 0.25-2.10). Conversely, the odds in the highest tertile of 16α-OHE1 showed a non significant risk increase (OR 1.76 95% CI 0.62-4.98). There was a suggestion of reduced Pca risk for men in the highest tertile of 2-OHE1to16α-OHE1 ratio (OR 0.56, 95% CI 0.19–1.68). The pooled estimates confirmed the association between an increased Pca risk and higher urinary levels of 16α-OHE1 (third vs. first tertile: OR 1.82, 95%CI 1.09-3.05) and the protective effect of an higher 2-OHE 1to16α-OHE1 ratio (third vs. first tertile: OR 0.53, 95% CI 0.31-0.90).

**Publications and Presentations**

The following paper detailing the results of the study has been prepared for *The Prostate* and is attached to this report.

*Urinary estrogen metabolites and prostate cancer: a case–control study and meta-analysis* Maddalena Barba, Francesca Sperati, Holger J. Schünemann, Li Yang, Sara Grioni, Saverio Stranges, Kim C. Westerlind, Michele Gallucci, Paola Muti

Dr. Muti has also published, or has in press, research on hormone-related cancer using a previously collected data set on hormone and prostate cancer (the dataset was originated from a previously DOD-funded study). She has submitted a paper for publication on the relationship between Indicators of Sexual and Somatic Development and Adolescent Body Size in Relation to Prostate Cancer Risk: Results from a case-control study.

In 2007-2008, Dr. Muti has published other papers on hormones and cancer, listed below:


Dr Muti has also presented new study results from this and other studies, at the Meetings of the American Association for Cancer Research as well as other conferences:

1) Speaker, “Health Research by Gender”, Women’s Health Day”, Naples, Italy, 7 March, 2007

2) “Can we use serum sex steroids to stratify the risk for breast and prostate cancer?” American Society for Clinical Oncology – Annual Meeting – Chicago, USA, June 2007

3) Chair of the meeting “The importance of health professionals and the patient at
the center in the National Health System”, IFO, IRCCS Regina Elena and San Gallicano, Rome, Italy, 21 June, 2007

4) Chair of the meeting “The biological testament in Cancer Patients”, Regina Elena Institute, Rome Italy, 5 July, 2007

5) Chair, “Evidence Based Decision Making In Daily Practice”, First workshop evidence-based health care for the surgical specialties Conference, organized by Regina Elena Institute in collaboration with Nicola’s Foundation, McMaster University and University of Toronto, Arezzo, Italy, 20-21 July, 2007

6) Speaker, “Intervention studies to reduce breast cancer risk”, National Academy of Medicine, Progress in the Management of Breast Cancer Conference, Modena, Italy, 13-15 September, 2007

7) Speaker, “Cancer and its impact in Italy and in the EU” Access to cancer drugs and allocation of resources”, Rome, Italy, 20 September, 2007


12)Speaker, “Patients affected with cancer and antidepressive treatment, the data on their efficiency” Depression and Cancer Corso ECM, IFO, Rome, Italy 10th December 2007


14)Speaker, “With the researchers” Presentation at the 2008 edition of Le Arance
15) Course Director and Moderator “2nd European Symposium on Liver-Directed Cancer Therapy using Microspheres”, Rome Italy 9th-10th February 2008

16) Speaker, “Bringing humanity into research and clinical assistance in oncology” Humanity and Today’s Health, Rome, Italy 3 March 2008


18) Speaker “The role of genes in the possibile prevention of cancer and the role of genes”, “I Sapori della Salute”, LILT, Rome, Italy 17 March 2008

19) Speaker “Vulvovaginal pathology HPV” conference, IFO, Rome Italy 11 April 2008

Chapters in Books


Letters (peer-reviewed)


Presentations at Meetings (Peer reviewed, selected) 2006/2007

(Presenter in bold. Some of these are published as abstracts, citation follows presentation)

1) Fuhrman BJ, Teter BE, Barba M, Byrne C, Cavalleri A, Grant BJ, Horvath PJ, Morelli D, Venturelli E, Muti P. Dietary Macronutrients and Equol Status as Determinants of Mammographic Density in a Sample of Postmenopausal Women from Western New York, USA. (poster presentation at the 2007 AACR annual meeting)
CONCLUSIONS

In summary, in the context of a still limited scientific panorama, our study and meta-analysis provide evidence supporting a differential role of the dominating estrogen hydroxylation pathway in prostate cancer development. The small sample size of our original study prevents us from drawing any clear-cut conclusion but the results of our meta-analysis including our studies provides significant evidence to support further studies to confirm this evidence. The appropriate use of quantitative methods might shortly allow the statistical pooling of our results with those from other investigations of good methodological quality addressing the same research question.
REFERENCES


Urinary estrogen metabolites and prostate cancer: a case–control study and meta-analysis

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\textsuperscript{5} Division of Endocrinology, Metabolism & Diabetes, University of Colorado at Denver and Health Sciences Center, Denver, Colorado, USA
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\textsuperscript{7} Scientific Direction, National Cancer Institute Regina Elena, Rome, Italy
Abstract

Objective: To investigate prostate cancer (Pca) risk in relation to estrogen metabolism, expressed as urinary 2-hydroxyestrone (2-OHE1), 16α-hydroxyestrone (16α-OHE1) and 2-OHE1 to 16α-OHE1 ratio.

Methods: We conducted a case–control study within the Western New York Health Cohort Study (WNYHCS) from 1994 to 2001. From January 2003 through September 2004, we completed the WNYHCS re-call and follow-up. Cases (n = 26) and controls (n = 110) were matched on age, race and recruitment period according to a 1:4 ratio. We used unconditional logistic regression to compute crude and adjusted odds ratios (OR) and 95% confidence interval (CI) of Pca in relation to 2-OHE1, 16α-OHE1 and 2-OHE1 to 16α-OHE1 by tertiles of urine concentrations (stored in a biorepository for an average of 4 years). We identified age, race, education, body mass index as possible covariates. After conducting an updated search of the literature which revealed no additional studies, we pooled the results from this study with those from a previously conducted case-control study using the DerSimonian-Laird random effects method.

Results: We observed a nonsignificant risk reduction in the highest tertile of 2-OHE1 (OR 0.72, 95% CI 0.25-2.10). Conversely, the odds in the highest tertile of 16α-OHE1 showed a nonsignificant risk increase (OR 1.76 95% CI 0.62-4.98). There was a suggestion of reduced Pca risk for men in the highest tertile of 2-OHE1 to 16α-OHE1 ratio (OR 0.56, 95% CI 0.19–1.68). The pooled estimates confirmed the association between an increased Pca risk and higher urinary levels of 16α-OHE1 (third vs. first tertile: OR 1.82, 95% CI 1.09-3.05) and the protective effect of an higher 2-OHE1 to 16α-OHE1 ratio (third vs. first tertile: OR 0.53, 95% CI 0.31-0.90).
Conclusions: Our study and meta-analysis provide evidence for a differential role of the dominating estrogen hydroxylation pathway in Pca development and encourage to the conduction of further studies.

Running Title

Estrogen Metabolism and Prostate Cancer Risk
Introduction

Prostate cancer (Pca) is the most frequently diagnosed malignancy and the second leading cause of cancer death among men in Western countries (1). Notwithstanding the importance of this tumour, its causes remain largely unknown. Age, family history, race and country of residence are the only established risk factors, but they explain only a small proportion of Pca incidence (2).

A considerable number of studies have addressed prostate sensitivity to androgens in relation to outcomes varying from normal prostate growth to benign and malignant diseases (3-5). However, the role played by estrogens in the pathogenesis of a wide spectrum of prostate physiologic and pathologic conditions is drawing increasing attention (6). In regards to Pca, experimental data from studies conducted in Noble (NBL) rats strongly suggest a critical role for estrogens in prostate carcinogenesis. Indeed, in NBL rats chronically treated with testosterone, the addition of estrogens is associated with a 100% incidence of prostate adenocarcinomas, whereas the administration of testosterone as a single agent produces Pca in approximately 30 to 40% of treated animals (7, 8). The estradiol plus testosterone treatment also induces acinar lesions that are similar to human prostatic intraepithelial, a well recognized preinvasive stage of adenocarcinoma (9).

Evidence is also mounting regarding the contribution of hydroxylated metabolites of estrone (E1) and estradiol (E2) to the overall estrogenic activity. The mutually exclusive hydroxylation of E1 and E2 at positions C-16α or C-2 leads to the production of either biologically active estrogens (16α-hydroxyestrone/estradiol) or derivatives with virtually no estrogenic activity (2-hydroxyestrone/estradiol), respectively (10-12). The different profiles in
terms of biological activity and genotoxic properties might have consequences in terms of Pca risk.

However, the overall body of evidence remains very restricted when considering estrogen metabolites in relation to Pca risk. We have previously investigated the ratio of 2- OHE1 to16α-OHE1 in a case-control study conducted in Buffalo, New York. Our results showed a reduced risk of clinically evident Pca in men with a lower 2-OHE1/16α-OHE1 ratio (13). Similar results from studies evaluating breast cancer, as another hormone dependent tumor, support this observation (14-18).

In the present case-control study, we have further tested the hypothesis that the pathway favoring 2- hydroxylation over 16α-hydroxylation based on urinary determinations of estrogen metabolites would be associated with a reduction in Pca cancer risk.

**Material and Methods**
From 1996 to 2001, 1961 men were enrolled in the Western New York Health Cohort Study (WNYHCS). A detailed description of the WNYHCS study design, participants characteristics and methods is available elsewhere (14). All participants provided informed consent; the Human Subjects Review Board of the University at Buffalo, School of Medicine and Biomedical Science approved procedures for protection of human subjects in the study.

At the time of recruitment, trained interviewers collected extensive data on demographics and life style during in-person interviews. The use of a standardized protocol allowed for the collection of anthropometrics. The study participants donated morning spot urine which was kept at – 80 °C until biochemical determinations.

From January 2003 through September 2004, we completed the Western New York Health Cohort (WNYHC) re-call and follow-up. For the purposes of the present case-control study (PROMEN II study), the re-call process included male participants meeting the following
inclusion criteria: age at recruitment between 50 and 85, baseline history negative for malignancies, cardiovascular diseases, and clinically defined type-2 diabetes, stored biological samples. On this basis, the re-call and follow-up process involved 1092 cohort participants. Among them, 52 were not eligible for medical reasons other than Pca, 46 had died from causes other than Pca, 22 had moved out of Erie and Niagara Counties, and 117 were not contactable by mail or phone. Among the remaining 855 study participants, 232 refused to join the study, 40 were scheduled but cancelled the appointment and 8 were still in-process at the end of the follow-up period. All of the cohort members in this group of non-participating subjects referred to being free from Pca in their telephone interviews. Thus, 575 participants joined the study, accounting for an overall participation rate of 67 % (575/855).

Pca cases were men who had been diagnosed with incident, histologically confirmed Pca over the time-frame between their recruitment in the WNYHC and the end of the follow-up period. Identification of Pca cases was based on the participants’ report at the re-call, which was subsequently validated by clinical records provided by the pertinent urologists. We identified and validated a total number of 41 incident prostate cancer cases.

The 534 control subjects were male members of the WNYHC who, based on their report, were free from clinically evident Pca at the time of diagnosis of the related case. The control status was validated based on serum PSA assessment on a blood sample donated at the time of recall. For specificity purposes, we adopted a PSA cut-off value of 4ng/ml (15). Among the study participants whose PSA level was higher than 4 ng/ml, we ultimately included in the control group only those who tested negative at the prostate biopsy. We requested and obtained the pertinent medical records from the urologists.
For each case, four control subjects were randomly chosen after matching for age (within a 3-year-range), race and date of recruitment. The independent variables of interest, namely 2-OHE1, 16α-OHE1 and the 2-OHE1 to 16α-OHE1 ratio, were available for 110 controls and 26 cases, thus we conducted the present analysis on 136 subjects.

**Hormonal Determinations**

For standardization purposes, we collected morning spot urine between 7:00 a.m. and 9:00 a.m. from all participants. We then transferred the aliquoted urine samples to the Eppley Institute, University of Nebraska Medical Center (UNMC), and stored them at -80 °C until analysis. Each analytical sample thawed only once prior to analysis. We handled urine samples identically and located them in the laboratory runs randomly. All laboratory personnel were blinded with regard to case-control status. We analyzed all of the study samples in duplicate. Two-milliliter aliquots of urine underwent partial purification by solid phase extraction (SPE) with a phenyl cartridge (Varian, Palo, Alto, CA) and ultraperformance liquid chromatography/tandem mass spectrometry (LC/MS-MS). We identified the analytes based on their retention time and tandem mass spectrometry. Standards of the catechol estrogens 2-OHE1(E2) and 16α-OHE1(E2) were purchased from Steraloids Inc. (Newport, RI).

Treatment of urine with glucuronidase/sulfatase led to marginal changes in the levels of estrogen metabolites and in many cases decreased the levels due to incubation for 8 h at 37 °C. To avoid the artifacts and errors introduced by maintaining the urine samples at 37 °C for 8 h, we carried out all the analyses without glucuronidase/sulfatase treatment.

We adjusted urine samples to pH 7 with 1 M NaOH or 1 M HCl. For method development and validation, 2-mL aliquots of charcoal-treated human urine samples were spiked with a total of 250, 500 or 1000 pg of the above compounds (final concentration 0.125, 0.25 and
0.50 pg/µL) and loaded onto the phenyl 100-mg cartridges pre-conditioned with CH$_3$OH and the loading buffer, 10 mM ammonium formate, pH 7. We eluted the compounds of interest from the cartridge after the washing and elution steps. This procedure led to enrichment of the target compounds after elution. We used Charcoal-treated urine (2 mL) in controls. The eluates from both the experimental and control samples were concentrated using a SpeedVac and lypholizer and assessed by LC/MS-MS. To determine the recovery of the standards by SPE method, we compared the corresponding concentrations of experimental and control samples. The recovery of 2-OHE$_1$ and 16α-OHE$_1$ was 97% and 101%.

We carried out the LC/MS analyses throughout a Waters Acquity ultraperformance liquid chromatography (UPLC) system connected with a high performance Quattro Micro triple quadruple mass spectrometer designed for LC/MS-MS operation. We performed the analytical separations on the UPLC system using an Acquity UPLC BEH C18 1.7 µm column (1 X 100 mm) at a flow rate of 0.15 ml/min. The gradient started with 80% A (0.1% formic acid in H$_2$O) and 20% B (0.1% formic acid in CH$_3$CN), changed to 79% A over 4 min, followed by a 6-min linear gradient to 45% A, resulting in a total separation time of 10 min. We then moved the elutions from the UPLC column to the Quattro Micro mass spectrometer.

The ionization method used for MS analysis was ESI in both the positive ion (PI) and negative ion (NI) mode with an ESI-MS capillary voltage of 3.0 kV, an extractor cone voltage of 3 V, and a detector voltage of 650 V. Desolvation gas flow was 600 l/h. Cone gas flow was 60 l/h. Desolvation temperature and source temperature were 200 and 100 °C, respectively. We performed the MS-MS in the multiple reaction monitoring (MRM) mode to produce structural information about the analytes by fragmenting the parent ions inside the mass spectrometer and
identifying the resulting daughter/fragment ions. We processed the resulting data and quantified
the estrogen metabolites using the QuanLynx software (Waters).

To calculate limits of detection, we injected various concentrations of the analyte to
LC/MS-MS, namely 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10, 25, 50 and 100 pg/µl. We considered the
injected amount that resulted in a peak with a height at least two or three times higher than the
baseline as the limit of detection. The limits of detection of 2-OHE$_1$ and 16α-OHE$_1$ were 18 fmol
and 349 fmol, respectively. Intra-assay coefficients of variation for 2-OHE$_1$ and 16α-OHE$_1$ were
3.2% and 3.0%, respectively. Inter-assay coefficients of variation were 1.9% and 3.5%,
respectively.

The intra- and inter-individual variability for 2-OHE$_1$, 16α-OHE$_1$ determinations and
their ratio expressed as intraclass correlation coefficients (ICCs) and lower limit of 95% CI (in
parentheses) were 0.70 (0.46), 0.63 (0.35) and 0.78 (0.62), respectively. We have previously
provided a detailed description of the procedures related to the reliability assessment (13).

**Statistical analysis**

We examined distributions for all variables of interest by determining the frequencies, mean,
median and measures of variance. To evaluate the statistical significance of the unadjusted
associations between case/control status and participants’ characteristics, we used either Fisher’s
exact tests or Pearson’s chi-square tests for categorical variables.

We standardized the 2-OHE$_1$ and 16-αOHE$_1$ urinary levels by total urinary creatinine.
We used unconditional logistic regression to compute crude and adjusted odds ratios (OR) and
95% confident interval (CI) of Pca in relation to 2-OHE$_1$, 16-αOHE$_1$ and the ratio of 2-OHE$_1$ to
16α-OHE$_1$ by tertiles of urine concentrations. We used the same models to test for significance
in trends of associations for any of the independent variables. We computed the cut-off points of
the previously mentioned tertiles based on the distributions of estrogen metabolites in control
subjects. We analyzed each independent variable separately. Based on the published literature,
we identified age, race, education level, BMI and waist-to-hip ratio as possible covariates and
tested them using regression models. Although none of them was a confounder for the
investigated associations, we included age in years in further analyses based on its biological
relevance in prostate carcinogenesis (2).

We verified several sources of potential bias. Because the exclusion of participants with
missing data for any of the two outcome variables could have introduced a source of bias in our
final sample, we examined data by subsets. Each of the two datasets included those men with no
missing data for either urinary levels of 2-OHE1 or 16-αOHE1. We then examined by case-case
and control-control comparison the characteristics of the 136 subjects (110 controls and 26
cases) with no data missing for any of the considered variables and those of the subjects (534
controls and 41 cases) who fulfilled our study eligibility criteria. Finally, we compared the
subjects in the latter category (575) to the 517 original cohort members who did not join the
study because of either not fulfilling the inclusion criteria, being lost to follow-up or not willing
to participate.
To date, no data specifically related to any of these three categories exist (i.e. co-morbidity data
pertinent to the WNYCS) exist. Thus, we considered these 517 male subjects as part of an
overall, although heterogeneous, category. As expected, the 517 males from the original cohort
who did not ultimately join our study showed statistically significant differences compared to the
575 included study participants. We analyzed these data using SPSS version 14.0 (SPSS, Inc.,
Chicago, IL).

Results
Table 1 shows the descriptive characteristics of the study participants. No significant differences emerge when comparing cases and controls by age, race, education, and anthropometrics.

In Table 2, we report crude and age-adjusted Pca risk estimates in relation to tertiles of urinary estrogen metabolites and their ratio. There was evidence of a risk reduction in the highest tertile of 2-OHE1; however our estimates did not reach statistical significance (OR 0.72, 95% CI 0.25-2.10). Conversely, the odds in the highest tertile of 16α-OHE1 showed an increased, but not statistically significant risk (OR 1.76 95% CI 0.62-4.98). Finally, the 2-OHE1 to 16α-OHE1 ratio showed a slight, non-significant reduction across tertiles (OR 0.56, 95% CI 0.19–1.68), in the highest tertile). When testing the independent variables of interest for significance in trends of associations, none of the models produced significant results.

Analyzing data by subsets including only one of the two outcome variables did not affect the study results at any level. From the case-case and control-control comparison, no significant differences emerged between the participants who had been included in the present analyses and those who had been excluded because of missing data items.

Given the small sample size of our study and the lack of statistical significance of our prior observations (13), a meta-analysis appeared to be a suitable instrument to derive a more stringent conclusion on the investigated association. We conducted a systematic search of the literature to identify additional studies published up to March 2008 which examined the association between estrogen metabolites and Pca risk. We searched MEDLINE (January 1966 onward) and EMBASE (January 1980 onward). An expert librarian designed a search strategy combining terms for estrogens, estrogen metabolites and prostate specific antigen (PSA) with terms for Pca. This yielded a total of 288 unique citations. Based on the titles and abstracts screening, none of the retrieved citations but our previously conducted case-control study (13)
was pertinent to the research question. We then combined the results from this and the previously conducted case-control study (13) using the DerSimonian-Laird random effects method and expressed the pooled estimates in terms of summary OR and 95%CI. We calculated $I^2$ to assess heterogeneity across study results applying the following interpretation for $I^2$ (J Higgins, personal communication): 0-50 = low; 50-80 = moderate and worthy of investigation; 80-100 = severe and worthy of understanding; 95-100 = aggregate with major caution (19). We used Revman 5.0 for statistical analyses.

Figure 1 shows the meta-analysis results. The pooled data concerned 122 Pca patients and 414 controls. The meta-analysis confirmed the association between an increased Pca risk and higher urinary levels of 16α-OHE1 (third vs. first tertile: OR 1.82, 95%CI 1.09-3.05) and the protective effect of a higher 2-OHE1 to 16α-OHE1 ratio (third vs. first tertile: OR 0.53, 95% CI 0.31-0.90). We found no statistically significant results for 2-OHE1. There was no evidence of heterogeneity ($I^2 = 0$, for any of the reported estimates).

Discussion

This study results suggest that the metabolic pathway favoring 2-hydroxylation over 16α-hydroxylation might be associated with a reduction in Pca risk. While these findings are not statistically significant, they appear consistent with those from a previously conducted, larger prospective study on the protective role of hydroxylated metabolites with virtually no estrogenic activity in the development of Pca (13). A meta-analysis of the results from these two studies, which was preceded by a systematic search of the literature showing no additional studies, produced significant evidence in support of the study hypothesis.

Our study has several strengths. The prospective design allowed for sample collection years before Pca diagnosis. On this basis, it is plausible that the observed differences in urinary
levels of estrogen metabolites by case-control status were not biased by any cancer-related hormonal activity in the diseased subjects group. In theory, the long-term effects of cryopreservation represent a potential source of variability because of the occurrence of sample degradation, but data from a previously conducted prospective study showed stability of estrogen metabolites over time (16). However, if any degradation effect exists, we might assume it as similar for cases and controls due to matching for date at recruitment. At the time of the WNYHC recall, we tested control subjects for potential presence of latent prostate cancer by serum analysis for PSA and, for those men whose PSA value exceeded the pre-defined cut-off, by prostate biopsy. This approach increases our confidence in the case-control definition and reduces the possibility for misclassification bias. We adopted several strategies to control for potential sources of hormone variability. In conducting the WNYHC recruitment and recall, we applied inclusion criteria claiming for the absence of pathologic conditions altering hormone metabolism (i.e. type 2 diabetes). We observed highly standardized conditions at sample collection, handling and assaying. All hormone determinations were performed at the end of the study, to reduce technical variability. We also evaluated the intra-individual variability of 2-OHE1, 16αOHE1 and their ratio in a previously conducted study (13). The resulting intra class correlation coefficients (ICC) indicated high reliability, thus reducing the chance that a measurement error might have affected the study results to a significant extent.

Our study also has several limitations. The sample size was very small, especially for cases, and none of the provided estimates reached statistical significance in the original study. Selection bias is another source of possible concern for several reasons. First, the participation rate was quite low (67%) and unfortunately we had limited information allowing a comparison between participating and non-participating subjects. Indeed, the lack of mortality or co-
morbidity data prevented us from characterizing those members of the original cohort who were excluded because of diseases other than Pca or death. The final comparison between the 575 men who joined the study and the 517 cohort members who did not showed significant differences. The exclusion of participants with missing data for either any of the outcome variables or any of the considered variables represents an additional, potential source of bias. Neither the analyses conducted by subsets including only one of the outcome variables nor the analyses performed by case-case and control-control comparison between subject with and without missing data items showed significant results.

We conducted a systematic search of the literature which revealed no further studies having investigated the association between estrogen metabolites and Pca risk and combined the available results in a meta-analysis. We found significant evidence supporting a protecting role of the metabolic pathway favoring 2-hydroxylation over 16α-hydroxylation in Pca development. This increases our confidence in the single studies’ results, which were anyways consistent, and might indicate that the lack of significance was mainly due to the quite limited sample size of the single studies.

Despite their historical use in prostate cancer treatment, our knowledge regarding the effects of estrogens on prostate, their role in cancer development and the mechanisms mediating their action as therapeutic agents is quite limited. The published literature mainly focuses on the effects of circulating estrone and estradiol in relation to prostate cancer risk, providing inconsistent evidence (17, 18, 20, 21). A wide variety of methodological issues ranging from the restricted sample size to possible bias introduced by uncontrolled sources of hormonal variability might provide a partial explanation to the cited inconsistency. It is also plausible that the discussed exposures have not been captured over periods comparable by degree of prostate
sensitivity to hormonal influences across the different studies. The lack of consideration for factors potentially relevant to the overall estrogenic activity, namely, hydroxylated metabolites of E1 and E2, might provide a further explanation that would integrate the aforementioned hypotheses.

The dominating hydroxylation pathway significantly affects the biological activity of estrogen metabolites. Indeed, 16α-OHE1 binds with high affinity the estrogen receptor and exerts a strong estrogenic action that leads to increased cell proliferation and DNA synthesis (22, 23). Moreover, the unique ability of 16α-OHE1 to bind DNA represents a solid basis for this metabolite’s genotoxic properties (12). Conversely, 2-OHE1 exerts a weak agonist effect on the oestrogen receptor and shows anti-angiogenic properties (24, 25).

Little epidemiologic evidence exists with regard to the hypothesis investigated in the present study. To our knowledge, only our and one other group investigated the pathway of 2-hydroxylation and 16α-hydroxylation in relation to prostate cancer (13, 26). Our previous study results support the association between elevated 2-OHE1 urinary levels and a reduced Pca risk (OR 0.83 95% CI 0.43-12.44), whereas elevated 16α-OHE1 urinary levels are associated with increased risk (OR 1.69 95% CI 0.93-3.06, p for linear trend 0.002). In their cross-sectional study, Teas et al evaluated the variability of the urinary levels of 2-OHE1 and 16αOHE1 in a sample of African–American men attending prostate cancer screening clinics and investigated any possible relation of these two metabolites with PSA. They reported an overall significant reduction in 2-OHE1 per each 1.0 ng/ml increase in PSA.

Further evidence for a role of sex steroid hormones in prostate cancer comes from studies focusing on the role played by estrogen metabolites in breast carcinogenesis. Several case-control and cohort studies show that women who metabolize a larger proportion of estrogens via
the 16α-hydroxy pathway may be at significantly higher risk of breast cancer compared to women who metabolize proportionally more estrogens via the 2-hydroxy pathway (16, 27-29). We observed a 40% breast cancer risk reduction in women whose 2-hydroxyestrone (2-OHE1) to 16α-hydroxyestrone ratio was in the highest tertile of the distribution compared to those in the lowest tertile (30).

In summary, in the context of a still limited scientific panorama, our study and meta-analysis provide evidence supporting a differential role of the dominating estrogen hydroxylation pathway in prostate cancer development. The small sample size of our original study prevents us from drawing any clear-cut conclusion but the results of our meta-analysis including our studies provides significant evidence in support of the investigated association and invites to the conduction of further studies. The appropriate use of quantitative methods might shortly allow the statistical pooling of our results with those from other investigations of good methodological quality addressing the same research question.
References


