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Accessibility
Pre-diagnostic circulating sex hormone levels and risk of prostate cancer by ERG tumour protein expression

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Background: Experimental studies have shown androgen receptor stimulation to facilitate formation of the TMPRSS2:ERG gene fusion in prostate cell lines. No study has tested whether higher pre-diagnostic circulating sex hormone levels in men increase risk of developing TMPRSS2:ERG-positive prostate cancer specifically.

Methods: We conducted a nested case–control study of 200 prostate cancer cases and 1057 controls from the Physicians’ Health Study and Health Professionals Follow-up Study. We examined associations between pre-diagnostic circulating levels of total testosterone, free testosterone, DHT, androstanediol glucuronide, estradiol, and SHBG and risk of prostate cancer by TMPRSS2:ERG status. TMPRSS2:ERG was estimated by ERG immunohistochemistry. We used multivariable unconditional polytomous logistic regression to calculate odds ratios (ORs) and 95% confidence intervals (CIs) for risk of ERG-positive (n = 94) and, separately, ERG-negative (n = 106) disease.

Results: Free testosterone was significantly associated with the risk of ERG-positive prostate cancer (OR: 1.37, 95% CI: 1.05–1.77), but not ERG-negative prostate cancer (OR: 1.09, 95% CI: 0.86–1.38) (Pdiff = 0.17). None of the remaining hormones evaluated showed clear differential associations with ERG-positive vs ERG-negative disease.

Conclusions: These findings provide some suggestive evidence that higher pre-diagnostic free testosterone levels are associated with an increased risk of developing TMPRSS2:ERG-positive prostate cancer.

Intraprostatic androgens play a critical role in normal prostate development and prostate carcinogenesis. Numerous studies have investigated associations between pre-diagnostic circulating sex hormone levels and prostate cancer risk, with mixed but overall null results (Roddam et al., 2008). Previous studies, however, have not considered the molecular heterogeneity of prostate cancer. In
particular, there is biological plausibility that sex hormones could differentially affect the development of prostate cancer with or without the somatic TMPRSS2:ERG gene fusion (Tomlins et al, 2005). Roughly half of prostate tumours harbour this fusion between the androgen-regulated gene TMPRSS2 and the oncogene ERG (Sextro et al, 2008; Tomlins et al, 2009; Rubin et al, 2011). Experimental work has shown that exposure of both malignant and non-malignant prostate epithelial cells to androgens can facilitate TMPRSS2:ERG formation (Lin et al, 2009; Mani et al, 2009; Bastus et al, 2010; Haffner et al, 2010). It is thus plausible that higher pre-diagnostic circulating sex hormone levels, especially of androgens, could increase the risk of developing TMPRSS2:ERG-positive prostate cancer. To address this question, we conducted a nested case–control study of 200 prostate cancer cases and 1057 controls assessing the associations between pre-diagnostic circulating levels of total testosterone, free testosterone, dihydrotestosterone (DHT), androstanediol glucuronide (a metabolite of DHT), estradiol, and sex hormone-binding globulin (SHBG) and risk of prostate cancer by ERG protein expression status (a marker of TMPRSS2:ERG fusion status).

MATERIALS AND METHODS

Study population. This study included the cases with known ERG protein expression status and all controls from two previously conducted nested case–control studies of pre-diagnostic circulating sex hormone levels and risk of prostate cancer (Gann et al, 1996; Platz et al, 2005). The first case–control study was nested within the prospective Physicians’ Health Study (PHS), a randomised, double-blind, placebo-controlled trial of aspirin and beta carotene among 22 071 US male physicians aged 40 to 84 at baseline in 1982 (Gann et al, 1996). The second case–control study was nested within the ongoing prospective Health Professionals Follow-up Study (HPFS), a cohort study of risk factors for disease among 51 529 male health professionals aged 50 to 75 years at baseline in 1986 (Platz et al, 2005). In both cohorts, all men were free of diagnosed cancer, other than non-melanoma skin cancer, at baseline. Participants in each cohort responded to a baseline questionnaire and follow-up questionnaires are mailed regularly (annually in the PHS and biennially in the HPFS) to update information on potential risk factors and to identify newly diagnosed illnesses. Prostate cancer diagnoses are self-reported and then confirmed through medical record review.

In the PHS, blood was collected from 68% of participants (n = 14 916) prior to randomisation. From among those who provided blood, every incident prostate cancer case occurring by March 1992 was matched to two living controls who had not reported a diagnosis of prostate cancer at the time of the case’s diagnosis (Gann et al, 1996). Controls were also matched on smoking status (never, former, current) and age within 1 year, except for two case patients who were over 80 at diagnosis for whom age was matched within 2 years. In total, 222 eligible cases and 390 eligible controls had plasma samples sufficient for analysis. In the HPFS, blood was collected from 18 018 participants free from prostate cancer between 1993 and 1995. From among those who provided blood, every incident prostate cancer case occurring by August 2000 was matched to one living control who had a prostate specific antigen (PSA) test after blood draw and had not reported a diagnosis of prostate cancer at the time of the case’s diagnosis (Platz et al, 2005). Additional matching criteria were year of birth (within 1 year), PSA test before blood draw (yes, no), timing of blood draw (midnight–before 0900 hours, 0900 hours–before noon, noon–1600 hours and after 1600 hours–before midnight), season of draw (winter, spring, summer, fall) and year of draw (exact). In total, 691 eligible case–control pairs had sufficient plasma samples for analysis.

The cases for this study initially included the 203 cases (PHS: 37/HPFS: 166) for whom tumour ERG expression status was available. We then excluded two T1a cases from the HPFS because such tumours are generally indolent and most susceptible to detection bias due to differential rates of surgery for benign prostatic hyperplasia. We additionally excluded 1 case and 17 controls who had a diagnosis of cancer other than non-melanoma skin cancer before the date of blood draw and 7 controls with a blood draw date after the date of their matched case’s diagnosis. The remaining 200 cases and 1057 controls comprised the analytical population for this study.

This study was approved by the Human Subjects Committee at the Harvard T.H. Chan School of Public Health and by Partners Health Care. Written informed consent was obtained from each subject.

Measurement of sex hormone levels. The measurement of sex hormone levels has been previously described in detail (Gann et al, 1996; Platz et al, 2005) and is outlined in Supplementary Table S1. In the PHS, plasma was assayed for total testosterone, DHT, estradiol, androstanediol glucuronide and SHBG in a single analytical run in December 1994. In the HPFS, measurement of sex hormone levels was completed in three waves: index date of diagnosis from blood draw to January 1996, February 1996 to January 1998, January 1998 to August 2000. Plasma was assayed for total testosterone, free testosterone, DHT (first two waves only), androstanediol glucuronide, estradiol and SHBG. For samples from both the PHS and the HPFS, cases and their matched controls were analysed together and laboratory personnel were unable to distinguish case, control and quality control samples.

Tumour tissue collection and assessment of TMPRSS2:ERG status. In both the PHS and HPFS, prostate tumour tissue has been collected from participants having undergone radical prostatectomy (RP) or transurethral resection of the prostate (TURP). Tissue microarrays have been constructed by taking three or more 0.6-mm cores of tissue from the primary tumour nodule or nodule with the highest Gleason grade.

We estimated presence or absence of TMPRSS2:ERG by immunohistochemical assessment of ERG protein expression as previously described (Pettersson et al, 2012). In short, ERG antisera (1:100, Clone ID: EPR3864, Epitomics, Inc., Burlingame, CA, USA) were applied to 0.5-μm TMA sections and visualisation of ERG was accomplished using the DAB substrate kit (Vector Laboratories Inc., Burlingame, CA, USA). A case was scored TMPRSS2:ERG positive if at least one TMA core had positive ERG staining within prostate cancer epithelial cells. Of cases positive for ERG on at least one core, 85% stained positive for ERG in all cores. Previous studies have shown ERG protein expression to be strongly correlated with TMPRSS2:ERG fusion status as assessed by other methods (Park et al, 2010; Chaux et al, 2011; van Leenders et al, 2011).

Statistical analysis. Because sex hormones were assayed in several batches, we adjusted for the effect of batch using methods previously described (Rosner et al, 2008). In brief, these methods involve regressing log-transformed hormone levels on batch. For all statistical analyses, batch-adjusted log-transformed hormone levels were modelled as continuous variables to maximise power, and we assessed 1 s.d. increases.

We first used unconditional binary logistic regression to estimate odds ratios (ORs) and 95% confidence intervals (CIs) for associations between sex hormones and risk of prostate cancer overall. We then used unconditional polynomyomous logistic regression, an extension of binary logistic regression that allows for nominal outcome variables, to study associations with three outcomes: ERG-positive prostate cancer, ERG-negative prostate cancer and controls. To maximise power, we combined the two
cohorts and adjusted for all matching factors used in either cohort. We also ran models additionally adjusted for body mass index (BMI) at blood draw and cohort. Missing data for model covariates (<10% for all covariates) were assigned to the mode value for categorical variables and the median value for continuous variables.

We conducted each of the analyses described for each of the hormones individually. Then, because estradiol and testosterone both bind to SHBG, we ran additional models for estradiol and, separately, testosterone that adjusted for SHBG, and a model including all three factors. To further explore findings from a previous study (Black et al., 2014), we also ran models of a ratio of estradiol to testosterone, both unadjusted and adjusted for SHBG.

In sensitivity analyses, we excluded participants with statistically extreme hormone levels according to the generalised Extreme Studentized Deviate many-outlier detection approach (Rosner, 1983).

Analyses were conducted using SAS version 9.2 (SAS Institute, Inc. Cary, NC). All tests were two-sided and \( P \)-values <0.05 were considered to be statistically significant.

### RESULTS

Characteristics of the 1057 controls, 106 ERG-negative cases and 94 ERG-positive cases are presented in Table 1. The mean time...
between blood draw and case diagnosis was 3.7 years. Cases in total were more likely than controls to have received a PSA test prior to blood draw. Among cases only, as previously reported (Pettersson et al., 2012), those who were ERG positive were younger at diagnosis and had higher stage tumours relative to those who were ERG negative.

Table 2 presents associations of sex hormones and risk of prostate cancer overall and by ERG status for 1 s.d. increases in the biomarkers. Increasing levels of total testosterone (OR per s.d.: 1.15, 95% CI: 0.98–1.36) and free testosterone (OR per s.d.: 1.21, 95% CI: 1.00–1.46, measured in the HPFS only) were associated with an elevated risk of prostate cancer overall. While DHT was not associated with risk of prostate cancer overall (OR per s.d.: 1.04, 95% CI: 0.87–1.24), its primary metabolite androstanediol glucuronide was positively associated (OR per s.d.: 1.16, 95% CI: 1.00–1.36). Neither estradiol (OR per s.d.: 1.00, 95% CI: 0.85–1.17) nor SHBG (OR per s.d.: 1.13, 95% CI: 0.95–1.34) were significantly associated with overall prostate cancer risk, but the latter did demonstrate a non-significant positive relationship.

In a polytomous model assessing the risk of ERG-negative and ERG-positive prostate cancer separately, free testosterone (measured in the HPFS only) was significantly associated with the risk of ERG-positive prostate cancer (OR: 1.37, 95% CI: 1.05–1.77), but not ERG-negative prostate cancer (OR: 1.09, 95% CI: 0.86–1.38) \( (P_{diff} = 0.17) \). None of the remaining hormones evaluated suggested differential associations with ERG-positive vs ERG-negative disease (all \( P_{diff} > 0.40 \)).

Adjustment for estradiol and/or SHBG did not materially alter the results for associations between total testosterone and prostate cancer. Likewise, adjustment for total testosterone and/or SHBG did not appreciably change the results for estradiol. The ratio of estradiol to testosterone was not associated with prostate cancer risk overall or by ERG tumour status.

No more than three outliers were detected for any hormone in any one assay batch. Results from sensitivity analyses excluding outliers were comparable to those from the main analyses. Similarly, additional adjustment for BMI, restriction to Caucasian men, exclusion of cases with ERG assayed in TURP specimens or tissue from an unknown source and exclusion of cases diagnosed within 1 year of blood draw did not materially alter the results (data not shown). The use of conditional logistic regression of matched sets only attenuated the results for free testosterone; the OR was 1.24 (95% CI: 0.84–1.83) for ERG-positive disease and 1.15 (95% CI: 0.82–1.61) for ERG-negative disease. On running analyses by cohort power was substantially reduced, particularly for the PHS (22 ERG-negative cases and 15 ERG-positive cases). Still, total testosterone was significantly associated with overall prostate cancer risk in the HPFS (OR: 1.25, 95% CI: 1.04–1.51), but not in the PHS (OR: 0.91, 95% CI: 0.62–1.33). No other results were significant in either cohort, except those for free testosterone (measured in the HPFS only).

**DISCUSSION**

In this nested case–control study of pre-diagnostic circulating sex hormones levels and risk of prostate cancer by ERG status, free testosterone was positively associated with risk of ERG-positive disease but unassociated with ERG-negative disease. None of the remaining hormones evaluated showed clear differential associations with ERG-positive vs ERG-negative prostate cancer. These findings provide some suggestive evidence that higher pre-diagnostic androgen levels are associated with an increased risk of developing TMPRSS2:ERG-positive disease specifically.

Experimental studies have shown that androgen receptor stimulation induces spatial proximity between TMPRSS2 and ER in both malignant and non-malignant prostate epithelial cells (Lin et al., 2009; Mani et al., 2009; Bastus et al., 2010). In addition, they have demonstrated that long-term (5 months) exposure to DHT alone (Bastus et al., 2010), or short-term (≤ 24 h) exposure to DHT plus gamma radiation (Lin et al., 2009; Mani et al., 2009), causes the formation of TMPRSS2:ERG. Other studies have investigated prostate cancer characterised by TMPRSS2:ERG status with respect to a polymorphic CAG repeat sequence in the first exon of androgen receptor that is associated with reduced...
transcriptional activity (Bastus et al, 2010; Figg et al, 2014; Mao et al, 2014; Yoo et al, 2014). Some (Bastus et al, 2010; Yoo et al, 2014), but not all (Figg et al, 2014; Mao et al, 2014), suggested that shorter repeat length may be specifically associated with the development of TMPRSS2:ERG-positive disease. The sum of these studies lends plausibility to the hypotheses that higher circulating pre-dormant sex hormones, and especially androgens, could be associated with the development of ERG-positive disease, and that the formation of TMPRSS2:ERG could be an initiating event or selected for during prostate tumour development. We found some evidence in support of these hypotheses, in that free testosterone trended towards stronger associations with ERG-positive disease. That the association with ERG-positive disease was present for free rather than total testosterone is noteworthy; free testosterone (i.e., testosterone that is unbound from SHBG or albumin) is a better measure of bioavailable testosterone for uptake into cells (Rosner et al, 2007). Still, all of the associations in our clinical data in humans were meager relative to evidence of associations between androgens and the induction of TMPRSS2:ERG in cell lines (Lin et al, 2009; Mani et al, 2009; Bastus et al, 2010; Haffner et al, 2010). Perhaps most conspicuous, we did not find any association between DHT and ERG-positive prostate cancer. It could be that higher levels of circulating androgens alone are insufficient to induce the DNA double-strand breaks that result in TMPRSS2:ERG formation; paracrine signalling or other factors that activate nuclear androgen receptor could be required (Pienta and Bradley, 2006; Taplin, 2007; Harris et al, 2009). The hypothesis that higher androgen levels increase the risk of TMPRSS2:ERG-positive prostate cancer is further challenged by evidence that CAG repeats are shorter (Buchanan et al, 2004) and circulating androgen levels are higher (Richard et al, 2014) in black than white men, but the prevalence of TMPRSS2:ERG-positive tumours is substantially higher in white (~50%) than black (~30%) men (Magi-Galluzzi et al, 2011; Rosen et al, 2012).

Many epidemiological studies have evaluated associations between circulating androgens and prostate cancer risk, with largely null results (Gann et al, 1996; Platz et al, 2005; Roddam et al, 2008). In the original case–control study from the PHS, investigators found that increasing total testosterone adjusted for SHBG was associated with prostate cancer risk (Ptrend = 0.004) (Gann et al, 1996). The original study from the HPFS did not return similarly significant results (Platz et al, 2005). Having consolidated the data from the two studies, we found, as expected, suggestive evidence that androgens may increase total prostate cancer risk, but with the exception of free testosterone, results were non-significant.

A limitation of this study is the relatively small number of cases assayed for ERG status, and that ERG protein expression was assessed rather than the TMPRSS2:ERG fusion itself. Cases assayed for ERG status, which were largely treated with RP, may not be representative of all men diagnosed with prostate cancer. In addition, our limited sample sizes precluded analyses of non-linear relationships and hindered meaningful interpretation of the results from the PHS (pre-PSA era) and HPFS (PSA era) separately. We were also unable to evaluate associations in non-Caucasian individuals. Still, our study was borne from a well-annotated prospective cohort with tumour tissue, plasma, and clinical data. Our analyses also adjusted for potentially important confounding variables, including age. Last, we were able to measure plasma hormone levels prior to diagnosis, thereby sidestepping any issues of reverse causation.

In summary, our study provides some suggestive evidence of associations between circulating free testosterone and risk of ERG-positive prostate cancer. Given the plausibility and experimental data in favour of the biological hypothesis, more studies should pursue this research question in the hopes of improving strategies for the primary prevention of disease.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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