A Large Study of *Androgen Receptor* Germline Variants and Their Relation to Sex Hormone Levels and Prostate Cancer Risk. Results from the National Cancer Institute Breast and Prostate Cancer Cohort Consortium

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Background: Androgens are key regulators of prostate gland maintenance and prostate cancer growth, and androgen deprivation therapy has been the mainstay of treatment for advanced prostate cancer for many years. A long-standing hypothesis has been that inherited variation in the androgen receptor (*AR*) gene plays a role in prostate cancer initiation. However, studies to date have been inconclusive and often suffered from small sample sizes.

Objective and Methods: We investigated the association of *AR* sequence variants with circulating sex hormone levels and prostate cancer risk in 6058 prostate cancer cases and 6725 controls of Caucasian origin within the Breast and Prostate Cancer Cohort Consortium. We genotyped a highly polymorphic CAG microsatellite in exon 1 and six haplotype tagging single nucleotide polymorphisms and tested each genetic variant for association with prostate cancer risk and with sex steroid levels.

Results: We observed no association between *AR* genetic variants and prostate cancer risk. However, there was a strong association between longer CAG repeats and higher levels of testosterone ($P = 4.73 \times 10^{-5}$) and estradiol ($P = 0.0002$), although the amount of variance explained was small (0.4 and 0.7%, respectively).

Conclusions: This study is the largest to date investigating *AR* sequence variants, sex steroid levels, and prostate cancer risk. Although we observed no association between *AR* sequence variants and prostate cancer risk, our results support earlier findings of a relation between the number of CAG repeats and circulating levels of testosterone and estradiol. (***J Clin Endocrinol Metab*** **95: E121–E127, 2010)**

Androgens are fundamental for the development and maintenance of the prostate gland and play a key role in prostate cancer biology. Early prostate cancer depends on androgens, and for the past 60 yr, the major therapeutic modality for metastatic prostate cancer has relied on decreasing androgen levels through surgical or pharmacological means (1). Based on the importance of androgens in prostate cancer biology, a long-standing hy-

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Abbreviations: AR, Androgen receptor; CI, confidence interval; SNP, single nucleotide polymorphism.

pothesis has been that circulating hormone levels affect prostate cancer risk, although a pooled analysis of worldwide data from 18 prospective studies (consisting of 3886 prostate cancer cases and 6438 controls) found no association between serum sex hormone concentrations and risk (2).

Androgenic action in the prostate is mediated by the androgen receptor (*AR*), which binds the two key androgens testosterone and dihydrotestosterone and activates expression of other androgen-responsive genes. *AR* mutations often lead to androgen insensitivity syndromes, and a highly polymorphic microsatellite $(CAG)_n$ located in exon 1 of the *AR* has been associated with *AR* transactivation where shorter repeats increase *AR* activity (3–5). Although some studies have found a positive correlation between CAG repeat length and serum testosterone levels (6, 7), several others have not been able to replicate this (8–10). A recent study in 2878 men found a significant correlation between longer CAG repeats and high circulating levels of testosterone and estradiol (11).

Association studies between germline genetic variation in *AR* and prostate cancer risk have been inconclusive. Most published studies have focused on the CAG repeat where shorter repeats have been suggested to increase risk, but this has not been consistent (12, 13). Only a few other studies have assessed other *AR* polymorphisms, and the two largest studies to date both found modest associations with aggressive prostate cancer (12, 14).

Recognizing the need for well-powered studies in genetic epidemiology, the National Cancer Institute (NCI) initiated the Breast and Prostate Cancer Cohort Consortium (BPC3) in 2003 (http://epi.grants.cancer.gov/BPC3/). The BPC3 consists of seven large cohorts from the United States and Europe and includes to date over 8800 patients with prostate cancer and 9000 matched controls. In this study, we evaluate the impact of inherited variation in *AR* on circulating sex steroid levels and prostate cancer risk. The CAG repeat and six single nucleotide polymorphisms (SNPs) were genotyped in 6058 prostate cancer cases and 6725 controls, for whom DNA was available at the time of this study.

Subjects and Methods

Study Population

The BPC3 and member cohorts have been described in detail elsewhere (15). In brief, the Consortium combines resources from seven well-established cohort studies: the American Cancer Society Cancer Prevention Study II (CPS-II) (16), the α-Tocopherol, β-Carotene Cancer Prevention (ATBC) Study (17), the European Prospective Investigation into Cancer and Nutrition Cohort (EPIC; comprised of cohorts from Denmark, Great Britain, Germany, Greece, Italy, The Netherlands, Spain, and Sweden) (18), the Health Professionals Follow-up Study (HPFS) (19), the Multi-Ethnic Cohort (MEC) (20), the Physicians' Health Study (PHS) (21), and the Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening Trial (22). These cohorts collectively include over 248,000 men who provided a blood sample.

Prostate cancer cases were identified through population-based cancer registries or self-reports confirmed by medical records, including pathology reports. The BPC3 data for prostate cancer consists of a series of matched nested case-control studies within each cohort; controls were matched to cases on a number of potential confounding factors, such as age, ethnicity, and region of recruitment, depending on the cohort. For the current analysis, prostate cancer cases were matched to available controls by age in 5-yr intervals, cohort, and country of residence for EPIC. Informed consent was obtained from all subjects, and each study was approved by the Institutional Review Boards at their respective institutions.

The current study was restricted to individuals who self-reported as Caucasian. Cases from other ethnic groups were contributed mostly from the MEC study, which has been published elsewhere (12). We had genotype data for a total of 6058 prostate cancer cases and 6725 controls. Data on the stage and grade of disease at diagnosis were collected from each cohort, where possible. A total of 1098 cases were classified as advanced (stage C or D at diagnosis or death due to prostate cancer), and 657 were classified as high-grade (Gleason scores 8–10 or equivalent, *i.e.* coded as poorly differentiated or undifferentiated).

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SNP selection and genotyping

Polymorphisms were originally selected based on data from Freedman *et al.* (12). Freedman and colleagues genotyped 32 SNPs, but the majority of the loci were monomorphic in populations of European ancestry. Therefore, we selected six SNPs that were polymorphic and adequately captured the haplotype variation in the region of interest. The mean pairwise r^2 for the 32 original SNPs with a minor allele frequency of at least 0.01 at the *AR* locus (in the phase II HapMap data release) captured by these six SNPs is 0.90 in the CEU HapMap population. Genotyping was done in four core laboratories (Harvard School of Public Health, Boston, MA; Strangeways Research Laboratory, Cambridge, UK; National Cancer Institute, Rockville, MD; and University of Hawaii, Honolulu, HI) using the fluorogenic 5′-endonuclease assay (TaqMan) with the ABI Prism 7900 (Applied Biosystems, Foster City, CA). TaqMan assays were designed and optimized for each SNP. Quality control was done by the manufacturer (Applied Biosystems) and by the laboratories of the Cohort Consortium for another 500 test reactions. Detailed assay information for each SNP is available on http://www.uscnorris.com/mecgenetics/CohortGCKView.aspx. For each SNP, sequence validation was done, and a 100% concordance was observed (http://snp500cancer.nci.nih.gov). The interlaboratory variation was assessed in each laboratory by running assays on 94 samples from the CEPH families, and the completion and concordance rates were above 99%. At each core laboratory, the internal genotyping quality control was done by genotyping 5 to 10% of blinded samples in duplicate or triplicate. Because *AR* is located on chromosome X where men are haploid, Hardy-Weinberg Equilibrium testing is not applicable.

CAG microsatellite genotyping was performed in three different core laboratories (Harvard School of Public Health, Boston, MA; National Cancer Institute, Rockville, MD; and University of Southern California, Los Angeles, CA). In brief, oligonucleotide primers flanking the CAG repeat were constructed, and genomic DNA was PCR amplified using fluorescently labeled primers. Products were analyzed by fragment analysis using an ABI 3730 capillary machine (University of Southern California, Los Angeles, CA), an ABI 3700 capillary machine (Harvard School of Public Health), and the ABI GeneMapper 3.0 (National Cancer Institute).

Plasma steroid hormone levels

Data on prediagnostic blood levels of androstanediol (n = 4851), testosterone (n = 4753), estradiol (n = 2076), and SHBG (n = 4796) were available in five of seven BPC3 cohorts (HPFS, PHS, ATBC, EPIC, and PLCO). The hormonal analysis was performed at different points in time as a part of individual studies for each cohort, *i.e.* the number of included cohorts for one plasma hormone indicates the number of laboratories used. Mean intrabatch coefficients of variation ranged between 4.1 and 18% for each lab (23). Methods for steroid hormones and SHBG have been published elsewhere (24–28).

Statistical methods

We tested the association between *AR* variants and prostate cancer risk using Wald tests from conditional logistic regression (stratified by study and age at diagnosis or selection as a control in 5-yr intervals). The CAG repeat was coded either continuously as 10-repeat units or categorically according to approximate quintiles in controls (≤19, 20–21, 22, 23–24, ≥25). To test association between *AR* SNPs or CAG repeats and plasma biomarker levels, we used Wald tests from standard multiple linear regression, adjusting for laboratory batch (nested within study), age at blood draw in 5-yr intervals, and case-control status. To ensure approximate normality and homoscedacticity across cohorts, hormone levels were log-transformed before analysis, and outliers were removed using Rosner's procedure (29). For all haplotype analyses, we included expected haplotype counts in the appropriate regression analyses and used the most common haplotype as the referent. Expected haplotype counts were calculated separately for each study to account for differences in haplotype frequencies and minimize potential for population stratification bias. All haplotypes with frequency less than 5% were combined in a single category. For subjects with no missing data, haplotyping was straightforward (because men are haploid on the X chromosome); for subjects with missing data, we used the expectation-maximization algorithm to calculate expected haplotype counts. Global tests of association between multiple haplotypes spanning *AR* and each outcome were calculated using likelihood ratios (30). All reported *P* values are two-sided and uncorrected for multiple hypothesis testing. We used the Cochran's Q statistic to assess effect measure heterogeneity across cohorts for individual SNPs, individual haplotypes, and the CAG repeat.

Results

A total of seven *AR* polymorphisms (one microsatellite and six SNPs) were genotyped in 6058 cases and 6725 controls. Characteristics of the seven nested case-control study populations are reported in Table 1. We performed association analyses across multiple phenotypes, including overall risk, tumor stage, grade, and hormone levels. Results are presented for the entire population, adjusted for age and cohort. Study-specific results are presented in Supplemental Tables 1–9 (published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org).

We tested associations between the CAG repeat and hormone levels (androstanediol, estradiol, SHBG, and testosterone). Both estradiol and testosterone levels were significantly elevated in men with greater CAG repeat length (estradiol, $P = 0.0002$; testosterone, $P = 4.73 \times 10^{-5}$) (Table 2). For both estradiol and testosterone, a 10 CAG repeat increment was associated with approximately 8% higher hormone levels. Three SNPs (rs6152, rs1204038, and rs1337080) were nominally associated with estradiol levels (Supplemental Table 4); however, given the number of tests performed, we cannot rule out the possibility of chance findings.

The range of CAG repeats was 9 to 36 in controls and 8 to 35 in cases. We found no association between the CAG repeat and prostate cancer risk based on a continuous model [odds ratio, 0.96; 95% confidence interval (CI), 0.85–1.08; *P*(trend) = 0.46, per 10 CAG repeat incre-

TABLE 1. Means and SD values for age, *AR* CAG repeats, and plasma hormone measurements by cohort and case-control status

	Controls			Cases		
	n	Mean	SD	n	Mean	SD
Age						
CPS-II	1177	71.9	5.14	1168	71.8	5.11
ATBC	1054	58.7	5.08	1048	59.2	5.25
EPIC	1115	61.5	6.19	734	60.9	5.95
HPFS	654	65.6	7.39	659	65.7	7.45
MEC	452	67.9	7.12	457	69.8	7.1
PHS	981	60.9	7.44	825	60.2	7.76
PLCO	1292	65.1	4.99	1167	65.4	4.95
CAG repeats						
CPS-II	1150	21.9	3.04	1147	21.8	2.84
ATBC	994	22.3	2.86	990	22.3	2.91
EPIC	1029	21.8	2.94	689	22	2.93
HPFS	616	21.9	2.94	617	21.8	2.97
MEC	426	22	2.94	408	21.7	3.05
PHS	915	21.9	3.01	782	21.9	3.01
PLCO	1272	21.8	2.93	1144	21.8	3
Androstanediol (ng/ml)						
Total	2566	7.11	5.02	2275	7.17	4.94
ATBC	96	3.09	1.83	68	3.67	3.01
EPIC	639	7.23	4.50	616	7.15	4.33
HPFS	648	6.33	5.40	653	6.60	5.47
PHS	363	7.03	3.25	235	7.37	3.18
PLCO	820	8.12	5.62	703	8.00	5.33
Estradiol (ng/ml)						
Total	1118	32.5	10.3	958	32.6	11.0
ATBC	104	28.8	8.83	68	29.7	9.25
HPFS	651	31.6	9.09	655	32.2	9.68
PHS	363	35.1	12.2	235	34.6	14.2
SHBG (nmol/liter)						
Total	2549	52.5	35.9	2247	53.5	37.3
ATBC	108	90.1	32.4	71	88.5	29.9
EPIC	604	46.6	20.3	582	44.7	19.1
HPFS	651	73.2	50.8	656	74.8	51.7
PHS	364	24.1	13.4	235	22.4	12.1
PLCO	822	48.2	22.5	703	47.6	23.4
Testosterone (ng/ml)						
Total	2529	5.00	2.09	2224	5.03	2.06
ATBC	106	6.22	1.96	71	6.44	2.40
EPIC	593	4.90	2.03	561	4.98	2.12
HPFS	650	4.82	1.71	655	4.88	1.80
PHS	363	4.90	1.74	234	4.84	1.58
PLCO	817	5.11	2.49	703	5.12	2.28

ment] (Table 3). Similarly, the categorical analysis did not reveal an association (Supplemental Table 6). Furthermore, the CAG repeat was not associated with either advanced-stage or high-grade cancer ($P = 0.10$ and $P = 0.63$) using either case-control analysis (Table 3 and Supplemental Table 6) or case-only analysis ($P = 0.52$ for high-grade *vs.* low-grade cancer and $P = 0.12$ for high-stage *vs.* low-stage cancer). None of the six genotyped SNPs was

TABLE 2. Association between *AR* CAG repeats and plasma hormone levels

Hormone	Controls	Cases	Mean difference (95% CI)[a]	r^2 (%)[b]	*P* value[c]
Androstanediol	2452	2167	1.86 (−3.84, 7.89)	0.0089	0.52
Estradiol	1059	898	8.89 (3.99, 13.8)	0.7167	0.0002
SHBG	2435	2142	3.51 (−0.68, 8.14)	0.0616	0.10
Testosterone	2419	2121	8.39 (4.23, 12.7)	0.3725	4.73×10^{-5}

[a] Percentage increase in levels per 10 repeat increase in CAG length.

[b] Percentage variance in trait explained by CAG repeat length.

[c] *P* value from linear regression, adjusted for cohort, batch (within cohort), prostate cancer case-control status, and age in 5-yr intervals.

TABLE 3. Association between *AR* CAG repeats and prostate cancer

Outcome	Controls	Cases	Odds ratio (95% CI)[a]	*P* value
All prostate cancer cases (*vs.* controls)	6402	5777	0.96 (0.85, 1.08)	0.46
High-grade cases (*vs.* controls)[b]	6402	611	1.07 (0.81, 1.42)	0.63
Advanced-stage cases (*vs.* controls)[c]	6402	900	0.82 (0.65, 1.04)	0.10

Odds ratios, CI, and *P* value are from conditional logistic regression, stratified on age in 5-yr intervals and study.

[a] Multiplicative change in odds of prostate cancer per 10 repeat increase in CAG length.

[b] Gleason grade 8 or greater.

[c] Stage C or D at diagnosis or death due to prostate cancer.

associated with overall prostate cancer risk or aggressive disease (Table 4). Haplotype analysis showed similar null results (data not shown). We did not observe any evidence for effect-measure heterogeneity at the 0.05 level.

Discussion

Given the importance of androgens in prostate cancer growth, key genes in the androgen pathway have been proposed to be involved in prostate cancer initiation. Over the past decade, a large number of studies have investigated the role of the *AR* CAG microsatellite polymorphism in prostate cancer development, but no consensus has been reached. The formation of the BPC3 provided a unique opportunity to uniformly study this hypothesis in the largest set of prospectively collected prostate cancer cases and controls to date. We found strong evidence that the number of CAG repeats does not alter prostate cancer risk in populations of European ancestry. Based on the literature, we tested different models (*e.g.* continuous *vs.* cut-point for the CAG repeat) as well as various phenotypic traits (disease aggressiveness), but it did not alter the results. The tagSNPs chosen to capture additional genetic variation in *AR* were also not associated with prostate cancer risk.

Our assessment of the relationship between circulating levels of sex steroids and CAG repeat length based on up to 4500 men showed longer CAG repeat lengths to be associated with significantly higher levels of estradiol and testosterone in agreement with a recent large study (11).

TABLE 4. Association between *AR* tag-SNPs and prostate cancer

SNP	Major/minor allele	n		Minor allele Frequency (%)		Odds ratio (95% CI)	*P* value
		Controls	Cases	Controls	Cases		
All cases (*vs.* controls)							
RS962458	A/G	6603	5967	7.04	6.90	0.98 (0.86, 1.13)	0.80
RS6152	G/A	6552	5966	15.1	14.6	0.97 (0.88, 1.07)	0.51
RS1204038	G/A	6644	5992	15.0	14.6	0.97 (0.88, 1.07)	0.51
RS2361634	A/G	6581	5949	6.53	6.86	1.06 (0.92, 1.21)	0.45
RS1337080	A/G	6652	5986	7.35	7.03	0.96 (0.84, 1.09)	0.52
RS1337082	A/G	6650	5996	19.9	19.5	0.97 (0.89, 1.06)	0.54
High-grade cases (*vs.* controls)[a]							
RS962458	A/G	6603	647	7.04	5.87	0.83 (0.59, 1.17)	0.29
RS6152	G/A	6552	647	15.1	14.2	0.93 (0.74, 1.18)	0.55
RS1204038	G/A	6644	647	15.0	14.4	0.95 (0.75, 1.19)	0.63
RS2361634	A/G	6581	640	6.53	6.56	1.01 (0.73, 1.40)	0.96
RS1337080	A/G	6652	649	7.35	6.32	0.85 (0.61, 1.19)	0.35
RS1337082	A/G	6650	649	19.9	18.8	0.93 (0.75, 1.14)	0.46
Advanced stage cases (*vs.* controls)[b]							
RS962458	A/G	6603	930	7.04	7.31	1.04 (0.79, 1.35)	0.80
RS6152	G/A	6552	926	15.1	15.3	1.02 (0.84, 1.24)	0.84
RS1204038	G/A	6644	933	15.0	15.1	1.00 (0.83, 1.22)	0.96
RS2361634	A/G	6581	930	6.53	6.34	0.97 (0.73, 1.28)	0.82
RS1337080	A/G	6652	934	7.35	7.71	1.05 (0.81, 1.36)	0.72
RS1337082	A/G	6650	931	19.9	19.0	0.94 (0.79, 1.12)	0.51

Odds ratios, CI, and *P* value from conditional logistic regression, stratified on age in 5-yr intervals and study. Odds ratio compares risk in carriers of minor allele to risk in carriers of major allele.

[a] Gleason grade 8 or greater.

[b] Stage C or D at diagnosis or death due to prostate cancer.

These findings are further supported by the observation that individuals with Kennedy's disease, a disease caused by dramatic expansion of CAG repeats, typically have elevated testosterone levels (31). Because longer CAG repeats have been associated with decreased *AR* activity (3–5), it is plausible that men with longer repeats have elevated testosterone and estradiol levels to maintain sufficient androgen action. The association between the CAG repeat and testosterone levels has long been debated, and data have been inconclusive. However, negative studies have been 4-fold to 10-fold smaller in sample size and might therefore lack statistical power to find a true correlation. That longer CAG repeats are associated with increased circulating testosterone and estradiol concentrations in this study, but not with prostate cancer, argues against an important direct role for circulating androgen levels in prostate cancer etiology. This is supported by a recent analysis from the BPC3 (23) as well as a recent pooled analysis of 18 cohorts (2). Whether circulating levels adequately reflect androgen action in prostate tissue levels requires further study, however.

The BPC3 includes participants from seven cohorts and is the largest investigation of the association between genetic variants in the sex steroid hormone pathway and the risk of prostate cancer to date, with 37 genes studied (15). A next important step is to jointly analyze these genes with respect to prostate cancer risk.

We have demonstrated that inherited variation at the *AR* locus is not associated with prostate cancer risk. The most likely explanation for earlier significant findings is statistical chance due to smaller sample sizes. We recognize that characteristics of prostate cancer cases have changed over time with the introduction of prostate-specific antigen testing, and with screening practices varying widely across cohorts within BPC3, we did not specifically test this hypothesis. However, our findings regarding cases with high-grade and high-stage disease (*i.e.* less likely to have been screen-detected) were in line with our overall results. Also, detailed treatment data were not included for these individuals, which prevented us from testing whether *AR* germline variants altered prostate cancer progression. A recent study, for example, observed that prostate cancer cases that received hormonal therapy and carried the variant allele of an *AR* promoter SNP, rs17302090, had an increased risk of dying from prostate cancer (32). Given the latter results and the importance of androgens in prostate cancer treatment, it will be of interest to further test *AR* polymorphisms in the context of clinical factors, such as response to androgen ablation therapy and time to progression after failure of hormonal treatment.

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