Canonical Androstenedione Reduction Is the Predominant Source of Signaling Androgens in Hormone-Refractory Prostate Cancer

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Abstract

Purpose: It has been recognized for almost a decade that concentrations of signaling androgens sufficient to activate the androgen receptor are present in castration-resistant prostate cancer tissue. The source of these androgens is highly controversial, with three competing models proposed. We, therefore, wished to determine the androgenic potential of human benign and malignant (hormone-naive and treated) prostate tissue when incubated with various precursors and examine concomitant changes in enzyme expression.

Experimental Design: Freshly harvested prostate tissue [benign, hormone-naive, and hormone-refractory prostate cancer (HRPC)] was incubated in excess concentrations of cholesterol, progesterone, DHEA, androstenedione, or testosterone for 96 hours, and steroid concentrations in the conditioned media measured by gas chromatography–mass spectroscopy. Changes in the expression of androgen synthetic and/or degradative enzymes were determined by expression microarray and qPCR. Significant changes were confirmed in an independent dataset.

Results: Of the precursor molecules tested, only incubation with androstenedione gave rise to significant concentrations of signaling androgens. Although this was observed in all tissue types, it occurred to a significantly greater degree in hormone-refractory compared with hormone-naive cancer. Consistent with this, gene set enrichment analysis of the expression microarray data revealed significant upregulation of 17HSD17B activity, with overexpression of the canonical enzyme AKR1C3 confirmed by qPCR in the same samples and in a publicly available expression dataset. Importantly, we found no evidence to support a significant contribution from either the "backdoor" or "5-α dione" pathway.

Conclusions: Reduction of androstenedione to testosterone by the canonical HSD17B AKR1C3 is the predominant source of signaling androgens in HRPC. Clin Cancer Res; 20(21); 5547–57. ©2014 AACR.

Introduction

Evidence accumulated over the past decade clearly indicates that continued activation of androgen receptor (AR)-mediated signaling is critical to the development and progression of castration-resistant prostate cancer (CRPC). This is supported by evidence of receptor upregulation and nuclear localization both in experimental systems and clinical samples, as well as the increased expression of AR-regulated transcripts (notably KLK3/PSA) with disease progression, and confirmed by the recent clinical success of second-line hormonal agents that continue to target AR signaling (1–6). It is also clear that despite the presence of castrate levels of testosterone in serum, sufficient concentrations of the two most potent AR ligands, testosterone (T) and/or dihydrotestosterone (DHT) are present in human CRPC tissue at levels sufficient to activate AR, with potent inhibitors of androgen synthesis producing dramatic clinical responses in this phase of the disease (4, 7). The origin of these androgens in human disease is unknown and highly controversial.

Under normal physiologic conditions, T, the most important circulating androgen, is synthesized de novo from cholesterol by Leydig cells in the testes by two parallel pathways (recently reviewed by Chang and Sharifi in ref. 8). In a common first step, C27 cholesterol is converted to C21 pregnenolone by CYP11A1-mediated cleavage of a C6 side chain on the inner membrane of mitochondria. In a series of reactions, pregnenolone is then converted to C17 androstenedione by 17α-hydroxylase/17,20-lyase (CYP17A1) and 3β-hydroxysteroid dehydrogenase/Δ5→Δ4 isomerase
Translational Relevance

It has been known for some time that castration-resistant prostate cancer tissue contains levels of signaling androgens sufficient to activate the androgen receptor, but the source of these steroids is highly controversial. For the first time, we have performed a comprehensive screen of the steroidogenic potential of human benign, hormone-naïve, and progressive hormonally treated prostate cancer tissue, and identified that reduction of androstenedione via canonical enzyme pathways is the predominant source of signaling androgens. Importantly, we found no evidence of significant contribution from the previously described "backdoor" or "5α-dione" pathway to androgen synthesis, in contrast with a body of work based on cell lines and xenograft models. These results have important implications for our understanding of continued androgen synthesis in human disease, as well as the rational design of future therapies.

(HSD3B), via the alternative intermediates DHEA or progesterone, depending upon the sequence of enzyme action. Androstenedione is then reduced to T by 17β-hydroxysteroid dehydrogenase (HSD17B), which may be further reduced to DHT in specific target organs that express 5α-reductase (SRD5A1/2). The weak androgen DHEA and its sulfated form DHEA-S are also synthesized and released by the adrenal cells in the zona reticularis, but must be converted to T and/or DHT in peripheral tissues.

Three competing, although not mutually exclusive, mechanisms have been proposed to explain the presence of significant concentrations of T/DHT in CRPC. The most widely accepted view is that these potent androgens are of significant concentrations of T/DHT in CRPC. The most widely accepted view is that these potent androgens are derived from the intratumoral conversion of weaker adrenal androgens based on normal physiologic pathways. The critical feature of this mechanism is that T is an essential element, either as an AR ligand in its own right or as an immediate precursor for DHT. Two alternative pathways to DHT that bypass T have been recently described: de novo steroidogenesis via the "backdoor pathway," as well as direct conversion of androstenedione to DHT via the "5α-dione pathway" (9, 10). The more contentious is the de novo mechanism, which proposes that CRPC cells develop the ability to generate steroids directly from cholesterol, with synthesis of DHT in a 7 to 8 step reaction that bypasses the canonical androstenedione–T axis. This entails conversion of pregnenolone to androstanediol via androsterone, and then directly to DHT by a mix of both canonical (CYP17A and HSD17B) and backdoor (RDH5 and SRD5A1) enzymes. In the more recently described "5α-dione" pathway, it is proposed that adrenally derived androstenedione is reduced to 5α-androstanediol by SRD5A1, and then converted directly to DHT by HSD17B (see Fig. 1).

Although there is significant overlap in the enzymes involved in each pathway, accurate knowledge of the exact mechanism involved is important for the rational design of more targeted inhibitors, with the potential for increased efficacy and fewer side effects than currently available drugs. Unfortunately, direct evidence to support any of the proposed mechanisms is lacking, with most of the data being derived from human cell lines or xenograft models, which may not accurately reflect the changes that occur in clinical disease. We were, therefore, interested in directly determining the ability of hormone-refractory prostate cancer (HRPC) to generate the putative signaling ligands T and DHT from a range of androgen precursor molecules, and correlate this with changes in pertinent enzyme expression.

Materials and Methods

Tissue incubation with androgen precursors

Following the Institutional Review Board approval, fresh prostate tissue was collected from patients with benign prostatic hyperplasia (BPH), hormone-naïve or HRPC undergoing transurethral resection (TURP) or radical prostatectomy for clinical indications. Patients with hormone-refractory disease had evidence of clinical progression with or without a rising PSA despite treatment with androgen-deprivation therapy (Table 1). No patient was being treated with a 5α-reductase inhibitor (finasteride and dutasteride) at the time of surgery. Collected tissue was placed in PBS in a specimen jar on ice, weighed, and then gently teased apart with scalpel and forceps under sterile conditions. At TURP results in significant surface charring, tissue samples for steroid assays were preferentially prepared from the center of larger prostate chips. The resulting minced tissue aggregates were then divided into seven approximately equal portions, reweighed, and six of the seven samples cultured in 2 mL of phenol red–free RPMI (Gibco) containing 5% charcoal-stripped serum and 1% penicillin/streptomycin in a 24-well plate (Falcon). The remaining sample of fresh tissue was snap-frozen for RNA analysis. To five of the six tissue-containing wells, 1 μL of a 20 μg/μL stock of testosterone, cholesterol, progesterone, androstenedione, or DHEA was added, whereas the final well contained no additive. To account for continued androgen production in the absence of exogenous precursors, steroid measurements in individual patients were standardized to this negative control. The addition of exogenous steroids was repeated in corresponding wells containing no tissue as controls. Because of limited tissue availability, only one technical replicate per patient was possible, and the mean (SD) weight of fresh tissue used in each steroid assay was 135 (97) mg. The plate was then incubated at 37°C in 5% CO2 for 4 days, and the contents of the wells then spun at 5,000 rpm for 5 minutes and the supernatant collected for hormone extraction. As androgen metabolism is a dynamic process, a time-course experiment may give a more comprehensive picture of the intermediate steroids generated from each precursor, particularly if short-lived. However, given the limited amount of tissue available as well as the number or precursors we wanted to test, we were limited to a single time point. Although many of the synthetic reactions occur within minutes or hours in enzyme kinetic studies...
and cell culture experiments (10, 11), similar experiments using xenografts and/or human tissue have shown continued accumulation of measurable androgens in tissue supernatants using various precursors out to 72 to 96 hours (9, 10). As tissue culture beyond 5 days in our hands resulted in a significant drop-off in cell viability (data not shown), we chose 96 hours as a "cumulative" time point to best assess the potential contribution of the "de novo" and 5α-dione pathways to DHT synthesis. Remaining tumor tissue was fixed in formalin, paraffin-embedded, and sections stained with hematoxylin and eosin for pathologic assessment.

Hormone extraction
The hormone extraction was carried out on 2 mL of the supernatant from the tissue incubation. The samples were

![Diagram](image)

Figure 1. Schematic outlining recognized pathways of androgen synthesis. The canonical pathway is unmarked, whereas the "backdoor" and "5α-dione" pathways are marked in gray and pink, respectively. Adapted from Locke et al. (9) and Chang and Sarfii (8).

<table>
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<th>Sample ID</th>
<th>Age</th>
<th>Disease</th>
<th>Gleason score</th>
<th>Mets</th>
<th>Clinical progression</th>
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<td>57</td>
<td>HRPC</td>
<td>5 + 5</td>
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<td>Yes</td>
<td>LHRH agonist</td>
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<td>HRPC</td>
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<td>Yes</td>
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<td>5 + 4</td>
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<td>4 + 4</td>
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<td>3 + 4</td>
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transferred into a 12 × 75-mm glass disposable culture tube (Kimble Chase), and 1 μL of a 20 μg/μL stock of 16,16,17, d3-testosterone was added to each sample as an internal standard. Ethyl acetate (Sigma-Aldrich) was added to the sample at a ratio of 1:1, the tube was then capped and inverted twice, the lid removed and the sample allowed to settle for 10 minutes before transferring the top ethyl acetate layer to a new labeled glass tube. A second ethyl acetate extraction was performed on the original sample and added to the first extraction. The tubes were then placed in a heating block at 60°C overnight and the sample dried, capped, and stored at 4°C. The dried samples were then sent to the National Measurement Institute (Sydney, Australia), where 50 μL of 4-ppm methyltestosterone was added before being dried using a TurboVap (Zymark) with nitrogen gas and a 40°C water bath. Of note, 50 μL of N-methyl-N-(trimethylsilyl)triﬂuoroacetamide was added before 2 μL was injected onto the gas chromatography–mass spectrometry (GC/MS; Shimadzu GCMS-QP2010 Plus with the AQC-20S autosampler) and individual steroid species identiﬁed using the method of Schanzer and Donike (12). At least three technical replicates of known standards containing 0, 200, and 400 ng of each of the 15 different hormones were detected; androsterone, androstenedione, androstenediol, 5α,3α-androstanediol, 5β,3α-androstanediol, 5α,5β-androstanediol, cholesterol, DHEA, DHT, progesterone, 17-OH-progesterone, pregnan-3α-ol-20-one, pregnen-3,20-dione, 17α-OH-pregnenolone, and testosterone were also analyzed per run to construct a calibration curve for each of the hormones detected. This curve was then used to determine the concentration of the unknown hormone.

RNA tissue extraction

Total RNA was extracted from snap-frozen TURP tissue using the miRNeasy Mini Kit (Qiagen). The tissue was kept at −20°C and handled with RNA-free instruments. No more than 50 mg of frozen tissue was added to 700 μL of QIAzol lysis reagent in a 14-mL round-bottom Falcon tube. The sample was then homogenized for 40 seconds using the TissueRuptor homogenizer (Qiagen), ensuring that the sample was uniformly homogeneous with no small particles of tissue remaining. The sample was then transferred to a 1.5-mL Eppendorf tube and incubated at room temperature (RT) for 5 minutes before adding 140 μL of chloroform, capping the lid and shaking vigorously for 15 seconds. The tube was allowed to settle at RT for 5 minutes before centrifuging at 8,500 rpm at 4°C for 15 minutes, after which the sample was separated into three phases: the upper colorless aqueous layer containing the RNA, a white interphase containing the DNA, and a lower red organic layer containing the proteins. The upper layer containing the RNA was transferred to a new 1.5-mL Eppendorf tube, and 525 μL of 100% ethanol was mixed in thoroughly by pipette. The sample was then transferred to an RNeasy mini spin column and centrifuged at 13,000 rpm for 15 seconds at RT before discarding the flow-through and adding 700 μL of buffer RWT and centrifuging for a further 15 seconds at 13,000 rpm at RT, discarding the flow-through. Of note, 500 μL of buffer RPE was added to the spin column and centrifuged at 13,000 rpm for 15 seconds at RT before discarding the flow-through and washing the column a second time with 500 μL of buffer RPE and centrifuging for 2 minutes at 13,000 rpm at RT to dry the membrane. The spin column was placed in a fresh collection tube and centrifuged at 13,000 rpm for 1 minute to remove any residual buffer RPE. The spin column was then transferred into a labeled Eppendorf tube and 37 μL of RNase-free water added to elute the RNA before centrifuging at 13,000 rpm for 1 minute at RT. The concentration and purity of RNA were then determined using the NanoDrop 3300, before running a sample on an RNA gel to check RNA quality.

Gene-expression microarray

The HumanHT-12v4 BeadChip (BD-103–0204; Illumina) was used for gene-expression microarray profiling. Briefly, 100 ng of total RNA was used as input for cDNA library construction with the IlluminaTotalPrep RNA Amplification Kit (AM11791; Applied Biosystems) as per the manufacturer’s instructions. Poly-A RNA was selected and reverse transcribed to single-stranded cDNA, converted to double-stranded DNA, and purified. The DNA was then transcribed in vitro overnight in the presence of biotinylated UTP and CTP to produce biotin-labeled cRNA. This cRNA was hybridized to the BeadChip over 16 hours. Following the addition of streptavidin-Cy3, the BeadChip was read using an iScan System (Illumina) and raw intensities were generated from laser-excited fluorescence.

qRT-PCR

For each sample analyzed, 200 ng of RNA, extracted from prostate tissue, was reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer’s instructions. qRT-PCR was performed using 1 μL of cDNA, 0.5 μL TaqMan primers (predesigned and commercially available from Applied Biosystems), 5 μL of TaqMan Fast Advanced Master Mix (Applied Biosystems), and made up to 10 μL volume per well with UltraPure distilled water (Gibco). Samples were run on a 384-well plate using a Viia7 PCR machine (Applied Biosystems) under the following conditions: UNG incubation at 50°C for 2 minutes; polymerase activation at 95°C for 20 seconds; denature at 95°C for 1 second; and anneal/extend at 60°C for 20 seconds. TaqMan primers (Applied Biosystems) to steroidogenesis-associated genes were as follows: CYP11A1 (cat no. Hs00897320_m1); HSD17B4 (Hs01069990_m1); AKR1C3 (Hs00366267_m1); RDH5 (Hs00161263_m1); SRD5A1 (Hs00602694_m1); and SRD5A2 (Hs00936406_m1).

Bioinformatics processing

BeadChip array analysis was performed using the limma package as part of Bioconductor in R (13). Raw intensities
that $P$ value in the list. This is repeated for all $P$ values in the list to derive a $q$ value for each test.

**Results**

**Androstenedione is the preferred precursor for intraprostatic testosterone production**

To determine the efficiency whereby various precursors undergo intraprostatic conversion to more potent androgens, we measured the concentrations of 15 different steroids in the conditioned media following incubation of fresh BPH, androgen-naive or HRPC tissue for 96 hours with excess cholesterol, progesterone, androstenedione, DHEA, and testosterone. We observed that only incubation with androstenedione led to the generation of significant concentrations of T productions across all tissue types, and appreciable quantities of DHT were identified only when T was the precursor (Fig. 2).

Notably, we did not identify any significant DHT (or T) production when prostate tissue was incubated with two precursors of the "de novo" pathway, cholesterol and progesterone, nor did we detect any significant production of...
DHT from DHEA or directly from androstenedione by the "5α-dione" pathway (Supplementary Fig. S1), despite evidence of both SRD5A (T → DHT) and 3αHSD (androstenedione → androsterone) activity in HRPC tissue. Small quantities of androsterone were identifiable in media from both benign and castration-resistant prostate tissue when incubated with cholesterol, suggesting that de novo synthesis may occur at a low background level, although there was no significant upregulation with hormone resistance. When analyzed by tissue type, HRPC was more efficient at converting androstenedione to testosterone than both benign tissue and androgen-naïve prostate cancer (Fig. 2 inset), although there was no significant difference in the ability to reduce T to DHT. Interestingly, T was more efficiently converted to androstenedione rather than DHT in both benign and castration-resistant prostate tissue, mirroring the results observed when androstenedione was used as a precursor, indicating preferential upregulation of HSD17B activity in these tissues. Consistent with previous reports, progesterone, DHEA, androstenedione, and T lead to increases in cholesterol production, particularly in hormone-resistant tumor tissue (16).

**HRPC is associated with a significant enrichment of HSD17B expression**

The synthesis of T and DHT from precursor molecules in androgenic tissue under normal physiologic conditions occurs via a well-established sequence of reversible reactions, although more recently a number of alternative pathways have been described that bypass this canonical pathway in CRPC. As androgen levels may be affected by both changes in synthetic and degradative enzyme expression, and given the number of possibly redundant pathways involved, to obtain an unbiased global estimate of the expression of potential genes of interest, we analyzed gene expression in the same benign, androgen-naïve, and HRPC specimens using the Illumina HumanHT-12 microarray. As shown in Fig. 3A, although the expression of a number of enzymes implicated in androgen synthesis or degradation in prostate samples measured using the Illumina HT12 BeadChip and grouped by disease. B, gene set enrichment analysis of the gene-expression data using custom gene lists as detailed in the Supplementary Tables, with the associated P and q values.
genes of interest was consistently higher in hormone refractory than in androgen-naive prostate cancer samples (HSD17B4, HSD17B7, and HSD17B12), the magnitude of measurement difference was small and did not meet significance using the stringent false discovery cutoffs. This is not surprising, given the small number of cases within each group, variance in expression, as well as the potential redundancy of activity across a number of different isoenzymes. We, therefore, performed GSEA using a number of prespecified gene lists as shown in Supplementary Table S1, and identified significant enrichment for HSD17B activity at a q value of <0.1 (Fig. 3B). This is consistent with the results of our culture experiments, as HSD17B isoenzymes are responsible for the reversible 17-keto reduction of androstenedione to T.

**HRPC is associated with overexpression of the canonical isoenzyme AKR1C3**

To confirm some of the findings identified using the expression microarray, we performed qPCR using RNA isolated from a subset of the same tissue samples, with at least 3 biologic replicates in each group. Given the limited amount of starting RNA available from the sample set, we focused on a small number of genes of interest, including the “backdoor” genes CYP11A1, HSD17B9 (RDH5), and SRD5A1, as well as the canonical 17β-hydroxysteroid dehydrogenase HSD17B5 (AKR1C3) and the alternative isof orm HSD17B4, which appeared to be consistently upregulated compared with naive tissue, and overexpression of which has previously been associated with poor outcome in prostate cancer (17). We found that the expression of AKR1C3 was significantly elevated in HRPC samples compared with both benign and androgen-naive samples, whereas RDH5 was actually significantly decreased in CRPC samples compared with benign tissue (Fig. 4). Interestingly, consistent with previous reports, the expression of SRD5A1 was upregulated in hormone-refractory tumors (18), whereas SRD5A2 levels were unchanged.

**AKR1C3 is overexpressed in hormone-refractory metastases**

It is clear that like many solid tumors, prostate cancers are molecularly heterogeneous, and it is possible that different mechanisms are preferentially active in tissue from alternative clinical sites. Because of the limited tissue availability from biopsy of metastases, for our steroidogenesis and expression assays we used tumor derived from TURP or obtained at prostatectomy, which clearly represents events occurring in localized disease. To determine whether similar changes in enzyme abundance occur in the metastatic setting, we downloaded and reanalyzed expression data from the Taylor cohort (29 matched normal prostate samples, 131 primary prostate cancers, and 19 metastases), which were interrogated using the Affymetrix Human Exon 1.0 ST array (19). On the basis of the clinical annotations provided, we probed the differences in expression between non–castration-resistant (lymph node metastases obtained at the time of planned prostatectomy ± neoadjuvant androgen deprivation) and hormone-refractory metastases (distant metastases ± prior hormonal treatment ± prior chemotherapy), in comparison with both localized prostate cancer and benign tissue. Consistent with our qPCR results, we saw no increase in the expression of the backdoor enzymes CYP11A1 and RDH5 in castration-resistant metastasis (Fig. 5). In contrast, although the expression of the AKR1C3 in non–castration resistance metastases was similar to both normal prostate and localized prostate cancer, expression in castration metastases was increased by 153%, 112%, and 148%, respectively. The expression of SRD5A1 was similarly highest in castration-resistance metastases, although a more incremental increase in expression was observed across the tissue types. In contrast, SRD5A2 expression was significantly decreased in metastatic lesions compared with localized prostate tumors regardless of hormonal status. No significant differences in CYP17A1, HSD17B1, HSD17B2, HSD17B3, HSD17B4, HSD17B6, HSD17B7, HSD17B9 (RDH5), HSD17B10, HSD17B11, HSD17B12, HSD17B13, HSD3B1, HSD3B2, or SRD5A3 expression between castration-resistant and non–castration-resistant metastases were observed (data not shown).

**Discussion**

Ever since concentrations of potent signaling androgens sufficient to activate the AR under experimental conditions were measured in CRPC tissue, there has been much speculation as to their source. The orthodox view is that they are generated by the intratumoral conversion of adrenal precursors using physiologic androgenic enzymes and reactions, culminating in the reduction of T to DHT. Recently, two alternative reaction pathways that bypass T as the immediate precursor of DHT ("de novo" and 5α-dione pathway) have been proposed to be important in human disease. In this study, which is the first to comprehensively address androgen production by human prostate tissue at distinct stages of disease progression, we have identified that the only significant source of androgen precursor in any tissue type is the intermediate steroid androstenedione, and that the predominant poten t androgen generated is T. Conversion of androstenedione to T was observed in both benign and malignant prostate tissue, suggesting that this is a normal physiologic pathway, although the generation of T was significantly more efficient in hormone-resistant compared with hormonenaive tumor. In particular, we did not identify any evidence of DHT production from the "de novo" precursors cholesterol or progesterone, nor did we observe direct conversion of androstenedione to DHT via the 5α-dione pathway, despite evidence within the assay of necessary enzyme activity.

Consistent with the results of our steroid assay, we observed upregulation of HSD17B genes, which are responsible for the reduction of androstenedione to T in CRPC samples on GSEA, and confirmed overexpression of the canonical substrate-specific 17β-hydroxysteroid dehydrogenase isoenzyme AKR1C3 by PCR. A similar finding was observed specifically in castration-resistant metastases in the Taylor dataset, strongly suggesting that this mechanism...
is not limited to localized tissue. AKR1C3 expression has been previously shown to be hormonally regulated, in that treatment of cell lines with the synthetic androgen R1881-suppressed transcript levels, whereas castration leads to its upregulation in xenograft models (20). In addition, AKR1C3 expression is consistently upregulated as measured in both global and gene level analysis of human castration-resistant compared with hormone-naïve prostate tissue, as well as by immunohistochemistry of corresponding tissue sections (21, 22). Taken together, these results strongly suggest that upregulation of AKR1C3-mediated conversion of androstenedione to T is the predominant source of potent androgens in CRPC. In contrast with previously reported castration-resistant LNCaP xenografts and consistent with previous reports of human tissue analysis, we did not find significant enrichment for enzymes implicated in the backdoor pathway (20).

Chang and colleagues have proposed, on the basis of their analysis of a variety of prostate cancer cell lines and two metastatic CRPC fresh tumor tissue samples, the existence of a 5α-dione intermediate pathway whereby androstenedione is converted eventually to DHT via the intermediate 5α-androstane (10). They demonstrated the accumulation of DHT from the androstenedione precursor.

Figure 4. Mean ± SEM log-fold concentration of selected gene transcripts in BPH (n = 3 or 4), hormone-naïve prostate cancer (CaP; n = 3 or 4), or HRPC (n = 3 or 4) compared with a standard curve and normalized to various housekeeping genes. P values refer to the significance of two-sided t tests between indicated groups.
Androstenedione Is the Preferred Androgen Source in Human HRPC

in a time-course experiment for both LNCaP and LAPC4 cell lines, however, this was not demonstrated for the two castrate-resistant patient tissue samples they examined with only the conversion of androstenedione to the intermediate 5α-androstane-3a,17β-diol, suggesting that reasonable levels of 5α-androstenedione were produced upon addition of androstenedione, which were then further catalyzed by 3α-HSD (AKR1C3/AKR1C1) and AKR1C3/17βHSD3 to the respective downstream metabolites. In fact, we observed that the concentrations of androsterone measured in culture supernatants mirrored that of T when androstenedione was used as a precursor, suggesting that reduction of 5α-androstane-3a,17β-diol to androsterone in the intact male prostate with little detectable levels of DHT observed (26). Genetic studies of mutant individuals have also confirmed the physiologic role of SRD5A2 with individuals with 5α-reductase deficiency in which pseudohermaphroditism and hypogonadism are exclusive-

Figure 5. The box plot of median expression ± 95% confidence intervals of selected gene expression in the Taylor dataset categorized by tissue type and hormone status of metastases. LNCaP xenografts (n = 1) are represented by a horizontal bar for interest only. Outliers are represented by single-dot points. Differences between groups were determined using independent two-sided t tests (*, P < 0.05).

variants of the AKR1C3 gene, and to date more than 67 SNPs in coding regions of the AKR1C3 gene have been identified, with 47 of these being nonsynonymous and potentially able to change the functional activity of the enzyme (23). Subtle changes in substrate specificity and/or catalytic activity of AKR1C3 variants may favor conversion of substrates down one particular pathway over other potential alternatives, which may account for the unexpected accumulation of androsterone in the primary prostate tissues we measured. It was also surprising that despite elevated expression of the T to DHT converting enzyme SRD5A1 in our hormone-resistant samples compared with the hormone-naïve and BPH samples, we did not observe any increase in DHT. Raised levels of SRD5A1 have been observed in numerous studies of CRPC tissue samples, although our results suggest that the raised levels of messenger RNA detected in these samples do not equate with raised functional activity (21, 24, 25). Interestingly, the paradoxical observation of elevated T levels, despite increased expression of the T converting enzyme SRD5A1, has long been recognized in CRPC tissue samples, although there is usually a concomitant decline in SRD5A2 expression similar to that demonstrated in the Taylor dataset (21, 25). Physiologically, SRD5A2 is the only significant form of 5α-reductase in the intact male prostate with little detectable levels of SRD5A1 observed (26). Genetic studies of mutant individuals have also confirmed the physiologic role of SRD5A2 with individuals with 5α-reductase deficiency in which pseudohemaphroditism and hypogonadism are exclusively mutants in the SRD5A2 gene but not in SRD5A1 (27). This suggests that the key regulator of T to DHT conversion in prostate tumor tissues is in fact SRD5A2, which consistently declines in castrate-resistant tissue, in part, due to lack of the well-known DHT feed-forward regulatory effect (28, 29). This would explain the relative accumulation of T
over DHT in castrate-resistant tissues despite a relative increase in SRD5A1 expression, in that SRD5A1 is unable to efficiently catalyze conversion of T to DHT in prostatic tissues, despite evidence of its ability to do so in cell lines (10). Supporting this is the observation that DHT was generated from precursor T across tissue types at a relative efficiency commensurate with SRD5A2, but not SRD5A1 expression. It is important to note, however, that we did not include metastatic tissue in our functional assays, and that much of the preclinical evidence supporting both the "backdoor" and 5α-dione pathways has been generated using cell lines derived from clinical metastases. Therefore, although the expression of genes involved in androgen synthesis in castration-resistant metastases is consistent with our observations in primary tumors, it remains possible that alternative pathways play a more prominent role in secondary sites of disease.

We note that appreciable concentrations of DHT were identified only when T was used as a direct precursor; however, in hormone-resistant tumor tissue, oxidation back to androstenedione was more efficient than reduction to DHT, suggesting that production of DHT in castrate-resistant tissues is an inefficient process. It has been recognized for some time that the normal T to DHT ratio of approximately 1:10 is reversed in castration resistance, with T being the predominant androgen identified. It is interesting to speculate as to whether this switch in relative abundance of AR ligands has functional consequences for disease progression or whether it is merely a consequence of it. Two large prostate cancer prevention trials, which have sought to manipulate the T to DHT ratios in the prostates of subjects, potentially suggest that there may be functional consequences of switching these ratios within the prostate. The prostate cancer prevention trial showed that finasteride, a selective SRD5A2 inhibitor, reduced the risk of prostate cancer by 25%, but among the tumors that were detected, there was a 27% increase in the number of those that had higher Gleason scores of 7 to 10 (30). Similarly, the REDUCE trial examined the incidence of prostate cancer detected on biopsy among men at increased risk for the disease treated with dutasteride, which, unlike finasteride, inhibits both isoforms of 5α-reductase (31). During years 3 and 4 of this trial, there was a significantly increased number (P = 0.003) of Gleason 8 to 10 tumors detected in the dutasteride group versus placebo (32). There are many competing explanations for the increased risk of higher grade tumors detected in both these 5α-reductase inhibitor trials; however, it remains plausible that the observed increases in high-grade disease are, at least in part, due to the relative accumulation of T over DHT, a state commensurate with the prostatic milieu associated with advanced castrate-resistant disease.

Collectively, our results add further credence to calls for a comprehensive orchestrated targeting of the AR axis at the initiation of endocrine therapy, rather than the sequential phasing in of such treatments as is commonly the practice today. Particularly pertinent to our results is the development of specific AKR1C3 inhibitors, which would be interesting adjunct treatments that might specifically interfere with the switch in T to DHT ratios in the prostate (33–37).

Disclosure of Potential Conflicts of Interest
I. Haviv reports receiving a commercial research grant from TYRNOVO and is a consultant/advisory board member for HAVIV GENOMICS. No potential conflicts of interest were disclosed by the other authors.

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