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PII: S0002-9440(18)30499-1

DOI: 10.1016/j.ajpath.2018.07.029

Reference: AJPA 2988

To appear in: *The American Journal of Pathology*

Received Date: 12 June 2018

Revised Date: 24 July 2018

Accepted Date: 30 July 2018

Please cite this article as: Huhtaniemi R, Oksala R, Knuuttila M, Mehmood A, Aho E, Laajala TD, Nicorici D, Aittokallio T, Laiho A, Elo L, Ohlsson C, Kallio P, Mäkelä S, Mustonen MVJ, Sipilä P, Poutanen M, Adrenals Contribute to Growth of Castration-Resistant VCaP Prostate Cancer Xenografts, *The American Journal of Pathology* (2018), doi: https://doi.org/10.1016/j.ajpath.2018.07.029.

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ADRENALS CONTRIBUTE TO GROWTH OF CASTRATION-RESISTANT VCaP PROSTATE CANCER XENOGRAFTS

Riikka Huhtaniemi [1,2*], Riikka Oksala [2*], Matias Knuuttila [1], Arfa Mehmood [3], Eija Aho [2], Teemu D Laajala [1,4,5], Daniel Nicorici [2], Tero Aittokallio [4,5], Asta Laiho [3], Laura Elo [3], Claes Ohlsson [6], Pekka Kallio [2], Sari Mäkelä [1,7], Mika VJ Mustonen [2], Petra Sipilä [1] and Matti Poutanen [1,6]

[1]Institute of Biomedicine, Research Centre for Integrative Physiology and Pharmacology, and Turku Center for Disease Modeling, University of Turku, Turku, Finland

[2]Orion Corporation, Orion Pharma, Finland

[3]Turku Centre for Biotechnology, University of Turku and Åbo Akademi University, Turku, Finland

[4]Department of Mathematics and Statistics, University of Turku, Turku, Finland

[5]Institute for Molecular Medicine Finland (FIMM), University of Helsinki, Helsinki, Finland

[6]Institute of Medicine, The Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

[7]Functional Foods Forum, University of Turku, Turku, Finland

*Equal contribution

Number of text pages: 18

Number of figures: 5

Number of Tables: 1

Number of Supplementary Tables: 1

Short running head: Mouse Adrenals Contribute to CRPC Growth

Funding: Supported by the Academy of Finland, Cancer Society Finland, Drug Research Doctoral Programme, Finnish Cultural Foundation, Sigrid Juselius Foundation, Swedish government, under the Agreement for Medical Education and Research, the Swedish Research Council, and University of Turku.

Disclosures: R.O., E.A., D.N., and M.V.J.M. are employees and R.H. and P.K. are former employees of Orion Corporation, Orion Pharma. Orion Pharma provided funding for this study.

Corresponding author: Matti Poutanen, PhD

Institute of Biomedicine, University of Turku, Kiinamyllynkatu 10, 20520 Turku, Finland

Phone: +358503660140

E-mail: matti.poutanen@utu.fi

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ABSTRACT

The role of adrenal androgens as drivers for castration-resistant prostate cancer (CRPC) growth in man is generally accepted; however, the value of preclinical mouse models of CRPC is debatable, since mouse adrenals do not produce steroids activating the androgen receptor. In this study, we confirmed the expression of enzymes essential for *de novo* synthesis of androgens in mouse adrenals, with high intratissue concentration of progesterone (P_4), and moderate levels of androgens, such as androstenedione, testosterone, and dihydrotestosterone in the adrenal glands of both intact and orchectomized (ORX) mice. ORX alone had no effect on serum P_4 concentration, whereas orchectomized and adrenalectomized (ORX+ADX) resulted into a significant decrease in serum P_4, and into a further reduction in the low levels of serum androgens (androstenedione, testosterone, and dihydrotestosterone), measured by mass spectrometry. In line with this, the serum PSA and growth of VCaP xenografts in mice after ORX+ADX was markedly reduced compared to ORX alone, and the growth difference was not abolished by a glucocorticoid treatment. Moreover, ORX+ADX altered the androgen-dependent gene expression in the tumors similar to that recently shown for the enzalutamide treatment. These data indicate that that in contrast to the current view, and similar to human, mouse adrenals synthesize significant amounts of steroids that contribute to the androgen receptor–dependent growth of CRPC.

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INTRODUCTION

Prostate cancer (PCa) is the second most common cancer and fifth leading cause of death from cancer in men worldwide[1]. At early stages of PCa, when the cancer is clinically localized, the disease is curable with surgery or radiation, whereas systemic therapies are needed for the management of advanced disease. Androgens, acting via the androgen receptor (AR) are essential for PCa growth [2]. Accordingly, androgen deprivation therapy (ADT), achieved by suppression of gonadal androgen synthesis via orchectomy (ORX) or chemically with gonadotropin-releasing hormone (GnRH) analogs, has been the first-line treatment of advanced and metastatic prostate cancer over 70 years. Although the initial response to these treatments is nearly complete, the majority of patients become resistant to ADT, developing a lethal castration-resistant prostate cancer (CRPC) [3]. In most cases, the CRPC growth involves reactivation of the AR signaling. This concept has been clinically proven with two recently approved treatments for CRPC. These include a non-steroidal AR antagonist (enzalutamide) that also inhibit the nuclear translocation of AR [4], and a selective CYP17A1 inhibitor (abiraterone acetate) blocking the critical step in androgen biosynthesis [5–7]. Also several other new treatments targeting directly the AR signaling pathway are under development [8,9]. Despite improved response ratios and overall survival with the new treatments, current therapies are not curative [8,10].

It is known that therapies leading to suppression of androgen action may lead to the development of AR pathway–related resistance mechanisms, including AR gene amplification [11] or overexpression [12], production of mutated forms of AR [13,14] or AR splice variants [15,16], and the induction of intratumoral androgen biosynthesis [17,18]. Approximately 90% to 95% of circulating androgens in men are expected to be produced in testis and the remaining 5% to 10% in the adrenal cortex [19]. Under ADT, normal and cancerous prostate tissue have shown to utilize adrenal-derived DHEA for

the local synthesis of T and dihydrotestosterone (DHT) [20,21]. However, the significance of adrenal androgens in the development of CRPC has been difficult to establish. Results have also shown that certain PCa cell lines (PC346 and VCaP) predominantly exploit adrenal precursors for androgen production, whereas others (LNCaP) utilize equally well both adrenal precursors and *de novo* synthesis [17,21,22]. On the other hand, in clinical CRPC specimens, only low intratumoral levels of enzymes of the first steps of steroid synthesis have been observed, but an up-regulation of enzymes essential for conversion of adrenal precursors to active androgens were reported, suggesting a central role for the adrenal precursors in androgen production [20,23]. Furthermore, the mutated AR (T878A) expressed, eg. in LNCaP cells, is also activated by the precursors of androgen production, including progesterone [24,25].

In contrast to human, murine adrenals do not produce large quantities of DHEA or its sulphate (DHEA-S), partially due to the low levels of CYP17A1 [26], and thus, the value of preclinical mouse models for studying androgen-dependency of CRPC has been questioned. In the present study, VCaP xenografts were used to study the role of murine adrenal steroids in CRPC growth. VCaP cells, originally established from a vertebral bone metastasis of a patient with CRPC [27], express wild type AR [27], and the VCaP xenografts in ORX mice demonstrate key characteristics of clinical CRPC, including up-regulation of intratumoral androgen biosynthesis, aberrant expression of both full-length AR and various AR splice variants, and responsiveness to AR antagonists [28,29]. The results show that ADX attenuates VCaP tumor growth in ORX mice, and results in reduction of intratumoral DHT, as well as down-regulation of androgen-dependent genes. Our data indicate that the steroid synthesis machinery in the mouse adrenal cortex contributes to the androgen-dependent growth of VCaP xenografts in castrated mice.

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MATERIALS AND METHODS

Cell lines

VCaP cells were obtained in 2006 from the American Type Cell Culture (ATCC, Manassas, VA) and were tested and authenticated by short-tandem repeat analysis in January 2014. All cell culture reagents were purchased from Gibco, Life Technologies (Carlsbad, Canada), except fetal bovine serum which was obtained from Hyclone Laboratories Inc. (Logan, UT).

Generation of VCaP xenografts

Athymic Nude male mice (Hsd:Athymic Nude-*Foxn1*^{nu}, Envigo, Gannat, France) at approximately five to six weeks of age, weighing between 20 to 30 g, were used in the studies. The mice were housed in individually ventilated cages under controlled conditions of light (12 h light /12 h dark), temperature (22 ±2 ºC) and humidity (55% ±15%) in specific pathogen-free conditions at the animal facilities of Orion Corporation, Orion Pharma, Finland. The mice were given irradiated soy-free natural-ingredient feed [RM3 (E), Special Diets Services, UK] and filtered, UV treated, tap water *ad libitum*. To maintain sodium balance, ORX+ADX mice had unlimited access to 0.9 % sodium chloride (Baxter, Deerfield, IL), instead of water.

Four to six days before inoculation, the cells were divided to confirm optimal growth potential in mice. At the day of inoculation, the cells growing at 80 % confluence were harvested using 0.05 % Trypsin-EDTA, centrifuged, counted, and suspended in the culture medium described above at a density of 26.6 x10^6 cells/mL. High protein concentration Matrigel™ (BD Biosciences, Bedford, MA) was then added (1:1), and a 150 µL aliquot of this suspension (2 million cells/mice) were inoculated s.c. to the right flank of each mouse by using a 25 G needle.

VCaP xenograft growth in intact, orchectomized, and both orchectomized and adrenalectomized mice

Development and growth of the VCaP tumors was monitored by measuring the tumor volume twice a week, and by detecting the serum concentration of prostate specific antigen (PSA) every 10 days. The volume of the tumors was calculated according to following formula:

$$W^2 * L / 2 \text{ (W = shorter diameter, L = longer diameter of the tumor).}$$

For PSA analysis, blood was collected from saphenous vein, and the PSA was measured with a time-resolved fluorometer (Wallac, PerkinElmer Analytical Life Sciences, Turku, Finland) as described previously [30]. Kim Pettersson (University of Turku, Finland) kindly provided reagents for the PSA assay.

Tumors were grown for seven weeks, until the mean volume of the tumors reached approximately 300 mm^3 (range 51 to 691 mm^3), and the mean serum PSA value was approximately 20 µg/L (range 1 to 93 µg/L). The experimental groups: intact (n=15), orchectomized (ORX; n=14), and both orchectomized and adrenalectomized (ORX+ADX; n=14) mice were allocated to study groups using our recently published algorithm [31], which matches the animals based on their baseline PSA concentration, tumor volume, PSA change from previous week, and animal weight to guarantee balanced and randomized allocation process. Following the ORX and ADX, the tumor volumes and the serum PSA were measured for eight subsequent weeks. At the end of the experiment the mice were sacrificed, and serum, adrenal glands, and tumors were collected for further analyses. Samples for steroid measurements and RNA isolation were stored at -80 ^{o}C after initial freezing in liquid nitrogen [29].

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VCaP xenograft growth in both orchectomized and adrenalectomized mice with corticosterone treatment

The VCaP tumors were initially established as described above in intact mice. When the tumors reached approximately 300 mm^3 (range 46-610 mm^3) the mice were allocated into three groups: ORX (n=13), ORX+ADX (n=13), and ORX+ADX with corticosterone treatment (n=13). ORX and ADX interventions were performed as described above, and after operations, corticosterone (Sigma-Aldrich, St.Louis, MO) was provided orally for four weeks (3 mg/kg/day) until the end of the study. The dose of corticosterone was selected based on literature [32] and on our previous studies. The appropriate range of used dose was also verified by measuring of plasma ACTH levels at the time of necropsy, ie, 24 h after last dose of corticosterone (Milliplex MAP Rat Pituitary Magnetic Bead Panel, Millipore Corporation, Billerica, MA). At the end of the study, adrenal glands and tumors were dissected and snap-frozen, blood samples were collected by cardiac puncture, and serum was separated and stored in -80 ºC.

National Animal Experiment Board of Finland have authorized the animal experiments with the licenses ESAVI/7472/04.10.03/2012 and ESAVI/1993/04.10.03/2011, and studies are performed according to the instructions given by Institutional Animal Care and Use Committees of University of Turku and Orion Pharma. The animals were kept according to the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health (NIH).

Measurement of intratumoral and serum steroids

Tumors were homogenized in sterile water using a TissueLyzer LT homogenizer (Qiagen, Venlo, The Netherlands), and intratumoral, intra-adrenal, and serum concentrations of P_4, A-dione, T, and DHT were measured using a previously described method applying GC-MS/MS [33]. Using mouse serum, the lower limit of quantitation (LLOQ) for P_4, A-dione, T, and DHT with the assay are 74

pg/mL, 12 pg/mL, 8 pg/mL, 2.5 pg/mL, respectively. Results under the LLOQ were calculated to be half of each LLOQ value to avoid overestimation of low values in the analysis. To make intratumoral steroid concentrations comparable to those in the serum, 1 g of tumor was considered equivalent to 1 mL of serum.

Quantitative RT-qPCR analyses and RNA-seq analyses

Total RNA for RT-qPCR and RNA-seq were extracted from tumor and adrenal gland samples using TRIsure (Invitrogen, Carlsbad, CA), and purified using RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. The primers and conditions for RT-qPCR analysis are shown in Table 1. The RNA-sequencing (RNA-seq) analyses of intact (n=5) and ORX (n=6) adrenal glands were performed at the Finnish Functional Genomics Centre, University of Turku and Åbo Akademi University and Biocenter Finland. The amount of 300 ng of total RNA was used according to Illumina TruSeq® Stranded mRNA Sample Preparation Guide. The samples were sequenced with Illumina HiSeq 2500 instrument (Illumina, San Diego, CA) using Hiseq v2 Rapid sequencing chemistry and 50 bp single-end read length.

The quality of the sequenced reads was checked using FastQC tool. STAR v2.5.0c [34] was used to align the reads to the mouse reference genome mm10, available at UCSC (Illumina iGenomes web site, San Diego, CA). The number of uniquely mapped reads associated with each gene according to RefSeq gene annotation was counted using the subread v1.5.0 R-package [35]. The RNA-seq data are available from the Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo/; accession number GSE117771). The downstream analysis of the data was performed using R version 3.2.2 [36] and its corresponding Bioconductor module 3.2 [37]. The count data were normalized for library size using the Trimmed Mean of M-values (TMM) method implemented in the edgeR R-package [38]. The normalized data were further transformed using the voom approach in the limma R-

package [39]. R package ROTS [40] was used for performing the statistical testing and false discovery rate (FDR) < 0.05 and absolute fold change > 2 was required for detecting the differentially expressed genes between the intact and ORX groups. The hierarchical clustering of the scaled normalized expression values of differentially expressed AR-regulated genes was performed using Euclidean distance and Ward's method, implemented in the R package pheatmap [41].

Immunoblotting

Tumor and adrenal gland samples were homogenized using a TissueLyzer LT and stainless steel beads (Qiagen, Hilden, Germany) in RIPA-lysis buffer containing the following: 150 mmol/l Tris-HCl, 1 % NP-40, 0.5 % sodium deoxycholate, 1 mmol/L EDTA, 1 mmol/L SDS, 100 mmol/L sodium orthovanadate (Sigma-Aldrich, St.Louis, MO), and cOmplete Mini protease inhibitor (Roche Diagnostics, Mannheim, Germany). AR was immunoblotted with standard techniques, as previously shown [28]. ImageJ software version 1.51K (NIH, Bethesda, MD) was used to compare and quantify the intensity of bands on scanned images of membranes.

Statistical analysis

The statistical tests were chosen depending on the results of the preliminary Shapiro-Wilk tests of data normality. Non-parametric Mann-Whitney, Kruskal-Wallis, and Dunn's multiple comparison tests were applied in RT-qPCR comparisons on single gene level, and to test the differences in the steroid concentrations in intact, ORX, and ORX+ADX mice, in comparison to vehicle and corticosterone-treated animals. Correlation of *Cyp17a1* and *Lhcgr* mRNA expression in adrenal gland was calculated using Spearman correlation coefficient. These univariate statistical analyses were performed using GraphPad Prism 7 software (GraphPad Software, San Diego, CA). The longitudinal analysis of PSA profiles between the interventions was performed using mixed-effects models that infer differences in population growth slope co-efficients over the whole time period of

the intervention [42], where the individual prognostic baseline variables were accounted for by incorporating them in the baseline animal matching [31]. The R statistical software (version 3.2.1) [36] together with the preclinical analysis R-package *hamlet* (version 0.9.5) were utilized in the longitudinal analyses [31]. *P*-value < 0.05 was considered statistically significant.

RESULTS

Growth of castration-resistant VCaP tumors is partially adrenal driven

Orthotopic VCaP xenografts in ORX hosts display the fundamental properties of CRPC, namely androgen-dependent growth and secretion of PSA, as well as AR overexpression and up-regulation of full-length AR and its splice variants [28]. In the present study, similar growth properties were confirmed for subcutaneous VCaP xenografts. As expected, ORX and ORX+ADX caused a transient repression of tumor growth rate and serum PSA concentrations (Fig. 1A, B). Notably, ADX delayed the relapse and reduced tumor growth in ORX mice. At the end of the study the tumor volumes in the ORX+ADX mice were smaller ($P < 0.001$) than those in the ORX group (Fig.1A). Similar pattern was observed in serum PSA concentrations (Fig. 1A). One week post ORX, PSA concentration reduced 6-fold, (from 20 µg/L to 3.4 µg/L), whereas 22-fold decline (from 20 µg/L to 0.9 µg/L) was seen in ORX+ADX mice. In the ORX group, serum PSA reached the pre-castration levels at about seven weeks, but in ORX+ADX group the increase in PSA levels was less evident and did not reach the pre-castration levels within the nine week–long study period (Fig. 1B). At the end of the study, PSA concentration in ORX+ADX mice was significantly ($P < 0.05$) lower compared to ORX mice Thus, these data show that ADX significantly reduces the growth of the castration-resistant VCaP tumors, indicating a contribution of adrenals to the CRPC xenograft growth in mice.

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The possible role of glucocorticoids in ADX response was next studied. Corticosterone replacement at physiological dose had no effect on the growth of VCaP xenografts in ORX+ADX mice (Fig. 1C). ORX, however, significantly ($P < 0.01$) increased the weights of adrenals (Fig. 1D), and as expected, ORX+ADX resulted into a marked increase in serum ACTH-level (26-fold higher compared to ORX), whereas a smaller change (7-fold compared to ORX, Fig. 1E) was observed in corticosterone-treated mice. These data confirm that the inhibition of VCaP xenograft growth by ADX is not due to the lack of adrenal glucocorticoids.

Mouse adrenals express enzymes involved in androgen production

Since the lack of glucocorticoids did not explain the inhibition of VCaP xenograft growth in ORX+ADX group, the expression of adrenal enzymes involved in sex steroid biosynthesis was analyzed by RNA-seq and RT-qPCR analysis (Fig. 2A, B, and D). Interestingly, RNA-seq revealed mRNA expression of 59 CYP, 16 AKR, and 62 SDR family enzymes. These include both classical steroid biosynthesizing enzymes, as well as several other enzymes whose role in steroid synthesis is yet to be defined, suggesting various possible pathways for androgen production. Some of these enzymes are known to be involved in early stages of steroid synthesis and, thus, affect both glucocorticoid and androgen production, whereas some are more specifically related to androgen production. Of these, the mRNA expression of *Akr1c6* (homolog for human *AKR1C4*), *Akr1c18*, *Akr1d1, Cyp17a1, Ugt1a1, Cyp11a1, Hsd3b1*, *Hsd3b2, Srd5a1,* and *Srd5a3* were confirmed by RT-qPCR and were shown to be induced 1.6 to 3736.6-fold by ORX ($P < 0.01$ to 0.001), with highest induction in the expression of *Akr1c18* (Fig. 2A, B). Furthermore, ORX resulted in marked up-regulation of luteinizing hormone/choriogonadotropin receptor (*Lhcgr*, 29-fold, $P < 0.005$), and significant up-regulation of the melanocortin 2 receptor (also known as adrenocorticotropic hormone receptor, *Mc2r*, 2-fold, $P < 0.001$) and *Ar* expression (2-fold, $P < 0.001$) in the adrenals (Fig. 2C). Interestingly, a close correlation between the *Cyp17a1* and *Lhcgr* mRNA expression was

observed (P < 0.001, Fig. 2E), suggesting that high circulating luteinizing hormone (LH) induces the early steps of the adrenal steroidogenesis in ORX mice. Top 30 up-regulated and down-regulated genes in the RNA-seq data in mouse adrenal gland are shown in Supplementary Table S1.

Mouse adrenals produce active androgens and their precursors, and contribute to sex steroid production in VCaP xenografts in castrated mice

The enzymatic machinery in adrenal glands, possibly involved in the androgen biosynthesis, prompted us to analyze the intra-adrenal steroid concentrations of intact and ORX male mice. Our data showed that, together with P_4, high concentrations (>1 ng/g tissue) of all main classical androgens (A-dione, T, and DHT) were present in the adrenals of intact mice. The mean concentrations of P_4, A-dione, T, and DHT were 4,367, 1.7, 3.0, and 1.5 ng/g, respectively (Fig. 3A). Thus, the P_4 concentration was over 1,000-fold higher than any of the quantitated androgens. In addition, high tissue-serum ratios for A-dione (84:1), DHT (94:1), and P_4 (50000:1) indicate local synthesis of these steroids in the adrenal glands (Fig. 3B). Interestingly, ORX had no effect on intra-adrenal A-dione, T, DHT, or P_4 concentrations. However, the adrenal-serum ratio of all these steroids increased significantly after ORX (Fig. 3B). These findings further support the hypothesis of intra-adrenal production of androgens in mice.

Notably, ORX alone had no effect on serum or intratumoral P_4 concentrations (Fig. 4A, B), whereas ORX+ADX resulted into 13-fold and 21-fold reduction in the P_4 concentration in the serum (Fig. 4A) and in the tumor (Fig. 4B) , respectively. This observation confirms that both serum and intratumoral P_4 mainly originates from the adrenals. ADX further reduced T and DHT concentrations in the serum of ORX mice (Fig. 4A), suggesting that murine adrenals indeed contribute to circulating androgen concentrations.

In line with our previous data [28], intratumoral P_4 and the androgen concentrations remained high in the castration-resistant VCaP tumors, despite the low serum steroid concentrations in ORX mice (Fig. 4B). Importantly, ADX decreased intratumoral A-dione, T, DHT, and P_4 concentrations in ORX mice (Fig. 4B). However, intratumoral synthesis of DHT is maintained after ADX, as tumor-serum ratio of DHT remains high. Taken together, the present data show the role of adrenals in the regulation of intratumoral androgen concentration in the VCaP xenografts in ORX mice.

Adrenalectomy affects androgen-dependent gene expression in the CRPC VCaP tumors

In line with our published data [28], the present study confirmed the up-regulation of mRNAs for full-length AR and AR splice variants by ORX (Fig. 5A). Interestingly, ADX further contributed to the over expression of AR in castration-resistant VCaP xenografts (Fig. 5A). At the protein level, however, no induction by ADX was observed (Fig. 5B), likely due to lower stability of the protein in the presence of only a low amount of the ligand [43,44]. ADX+ORX also induced the expression of certain enzymes involved in DHT synthesis in the tumors, including AKR1C3 and SDR5A1 (Fig. 5C). In addition, the expression of set of genes previously [29] identified to be regulated by antiandrogens in castration-resistant VCaP xenografts was analyzed. Of these genes, *SYLT2* and *NOV* were up-regulated and *FKBP5* and ST6GAlNAcl down-regulated after ADX+ORX (Fig. 5D). Overall, the effect of ADX in castration-resistant VCaP xenografts was similar to that shown for antiandrogens (enzalutamide and apalutamide) [29]

DISCUSSION

Xenograft models generated using PCa cells and clinical tissue specimens are widely used [45–47] for studying PCa biology and drug responses. The relevance of mouse CRPC models has, however,

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been questioned due to species differences in extragonadal sex steroid production [17,48]. In humans, weak androgens DHEA and DHEA-S, produced by adrenal glands, are recognized as precursors for the intratumoral synthesis of T and DHT [49]. In contrast, production of DHEA and DHEA-S by murine adrenals has been considered negligible, and, thus, having no significant contribution to local androgen biosynthesis in CRPC xenografts [26]. In the present study, this view was challenged by using up-to-date steroid profiling methodology, and evidence was provided for the significance of murine adrenal steroids in the regulation of CRPC xenograft growth.

VCaP xenografts in ORX mice exhibit several of the key characteristics of clinical CRPC, including castration-resistant growth, intratumoral androgen biosynthesis, marked expression of both full-length AR and various AR splice variants, and responsiveness to AR antagonists [28,29]. The role of adrenal steroids has not been addressed in the previous VCaP xenograft studies, possibly due to the presumption that murine adrenals lack the enzymatic machinery needed for the production of androgens. Our present results, however, show that ADX attenuates VCaP tumor growth and serum PSA in ORX mice, indicating that adrenal steroids contribute to the growth of castration-resistant VCaP xenografts. In line with that previously shown for VCaP cells after enzalutamide treatment *in vitro* [50], our RNAseq data indicated the induction of glucocorticoid receptor (GR) after ADX. Importantly, even if data have suggested that corticosteroids might promote the growth of antiandrogen-resistant prostate tumors [50], the tumor inhibitory effect of ADX on VCaP xenografts *in vivo* was not abolished by a treatment with the physiological dose of corticosterone. Thus, the effect of ADX on VCaP xenograft growth is not explained by the lack of glucocorticoids or reduced general well-being of the host. Furthermore, due to their low binding affinity, the amount of androgens present in the tumors likely do not activate the low level of GR present in the tumors.

The present study demonstrates that mouse adrenals produce biologically significant amounts of T and DHT, and contribute to the circulating levels of these androgens, especially after ORX. RT-qPCR analyses were in line with the steroid measurements, showing that murine adrenals express some of the key enzymes that selectively contribute to androgen synthesis (*Hsd17b3, Akr1c6, and Hsd17b6*) [48]. The discrepancy between previous studies [26] and our current results regarding murine adrenal androgen production may, at least partly, be explained by methodological issues. A highly sensitive mass spectrometry–based method was used to measure the intratissue and serum steroid concentrations [33], as against the less sensitive assays used in the previous studies. In line with earlier data [48], it was also observed that ORX caused a marked induction in the expression of adrenal LH receptor (*Lhcgr*) expression. Interestingly, a strong correlation was observed between the expression of *Lhcgr* and *Cyp17a1*, encoding for a key enzyme necessary for the synthesis of all steroid hormones in the adrenal cortex. As ORX increases circulating LH levels [51], these data suggest an auto-regulatory loop for LH to promote the early steps of steroidogenesis in the murine adrenals. Also in human, bilateral ORX increase serum LH levels [52]. Moreover, it has been shown that human adrenal gland express LH receptor in the adrenal cortex cells in the reticular layer [53], it was speculated that GnRH analog treatment may reduce adrenal steroidogenesis via suppressing the circulating LH. Currently, ADT is mainly achieved by chemical castration by GnRH analogs. However, cumulating evidence suggests that long term chemical castration is associated, for example, with higher risk of bone fractures and cardiovascular events compared to ORX [54]. Thus, there has been discussion on whether surgical castration should be favored over the GnRH analog treatment [54–56]. These data suggesting that high LH level after ORX is activating adrenal steroidogenesis would, however, favor the use of GnRH analogs in the treatment of PCa patients.

The correlation between intratumoral and circulating androgen concentrations indicates a systemic contribution to intratumoral androgen levels. In addition, in ORX mice, circulating P_4 is also a

likely precursor for the local androgen production in castration-resistant VCaP tumors. Previous studies have reported P_4 in the circulation in mice after ORX [17,33] but its origin has not been well established. Some studies suggest that production of P_4 in the tumors is the main source of the circulating P_4 in PCa xenograft bearing ORX mice [22,57], whereas the current data indicate a major role for adrenal P_4 production. It is well established that the human AR with the T878A mutation is strongly stimulated by P_4 [24], and there is some evidence to suggest that P_4 may induce AR action under the conditions of high AR expression [2,58]. As increased levels of AR are present in CRPC tumors [59], it is possible that also non-classical, low affinity ligands, such as P_4, can stimulate AR. Nevertheless, P_4 is only a very weak agonist for the wild-type AR (AR-FL), and based on the present data, i can be concluded that DHT, present at substantial concentrations in castration-resistant VCaP xenografts, is the main driver of intratumoral AR signaling in the ORX and ORX+ADX mice. Notably, P_4 may serve as a substrate for steroid 5α-reductases (SRD5A) in the backdoor pathway of DHT synthesis that bypasses the need of T as an intermediate [60]. VCaP xenografts grown in ORX+ADX mice express SRD5A enzymes, and the intratumoral P_4 and DHT levels are comparable to those in tumors from intact animals. This observation, together with a significant decrease of intratumoral T after ORX, and a further reduction after ADX, support the hypothesis of adrenal P_4 as a precursor for intratumoral DHT synthesis via the backdoor pathway. Furthermore, the possible contribution of novel androgens, such as the 11-keto-T and 11-keto-DHT [61], to the growth of VCaP xenografts in ORX+ADX hosts remains to be determined.

In line with clinical data [62], our previous study confirmed that ORX induces the expression of full-length AR and its splice variants [28] in VCaP xenografts, and that antiandrogen treatment further stimulates AR expression in castration-resistant VCaP tumors [29]. The present study shows that the effect of ADX, mimics the effect of antiandrogens, as regards the up-regulation of AR expression and stimulation of intratumoral DHT synthesis [29]. This result suggests that attenuation of tumor

growth by ADX is a result of inhibition of androgen action. Moreover, ADX and enzalutamide [29] modify the expression of androgen-dependent genes (*NOV*, *FKBP5*, and ST6GAlNAc1) in a similar manner, which further supports the idea of the anti-androgenic effect of ADX on the growth of VCaP tumors in ORX hosts.

Taken together, in contrast to the current view, we have demonstrated that adrenals of intact and ORX mice synthesize biologically relevant levels of active androgens, as well as precursors of androgen synthesis, such as P_4. As a result, murine adrenals significantly contribute to the growth of VCaP xenografts in ORX mice. Thus, mice resemble men more closely than previously thought, in terms of the relevance of adrenals in producing androgens and their precursors to be further synthesized in peripheral tissues and tumors. In conclusion, VCaP xenograft model resembles the clinical CRPC [63], and is an appropriate model to study androgen-dependent growth of CRPC and the mechanisms involved therein. Furthermore, the complete blockade of adrenal steroid synthesis, including all potential precursors for DHT, may be an additional option for prostate cancer patient.

ACKNOWLEDGEMENTS

We thank the personnel at the Institute of Biomedicine and Turku Center for Disease Modeling for skillful assistance; the staff at Orion Pharma for collaboration: Kati Raepalo, Timo Leino, Taina Kirjonen, Jenni Mäki-Jouppila, Satu Orasniemi, and Jenni Airaksinen; and Kim Pettersson, University of Turku, Finland, for providing reagents for the PSA assay.

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FIGURE LEGENDS

Fig. 1. Combined orchectomy (ORX) and adrenalectomy (ADX) attenuates VCaP tumor growth more efficiently than ORX alone, measured by serum PSA concentration (**A**) and tumor volume (**B**). **C:** Glucocorticoid (corticosterone) treatment does not abolish the effect of ADX on tumor growth in ORX mice. **D:** The relative weight of adrenal glands of ORX mice is increased compared to intact mice. The mean weight was 0.41 g (median 0.39 g, range 0.20 to 0.86 g) in ORX group, and 0.30 g (median 0.28 g, range 0.18 to 0.60 g) in intact group. Measurements were performed at the end of the study, 63 days after ORX or with intact 84 days after cell inoculations. **E:** Serum ACTH concentration in ORX+ADX group is partially normalized by corticosterone treatment. Measurements were performed at the end of the study, 30 days after ORX and ORX+ADX. The data shown in **A**, **B**, and **C** represent mean±SEM and data in **D** and **E** represent median and range. The statistical tests utilized were the following: Longitudinal mixed-effects models, as presented in [31], were applied for data presented in **A** and **B**. Kruskal-Wallis with Dunn's post hoc test at sacrifice was used for the data in **C**. Mann-Whitney U test was applied for the data in **D** and **E**. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Fig. 2. A: Relative mRNA expression of the top five most up-regulated enzymes (**A**) and other enzymes (**B**) related to steroid synthesis in adrenal gland of intact (n= 11, white bars) and orchectomized (ORX; n=11, gray bars) male mice. **C:** Expression of LH receptor (*Lhcgr*), the receptor for ACTH (*Mc2r*), and androgen receptor (*Ar*) in intact and ORX mice. **D:** Heat map of hierarchical clustering of the differently expressed enzymes in intact (n= 4) and ORX (n=5) mice adrenal gland. **E:** Spearman correlation between the expression of *Cyp17a1* and *Lhcgr*. All measurements were performed at the end of the study, 63 days after ORX or with intact 84 days after cell inoculation. Data in **A** and **C** are expressed as mean±SEM. RT-qPCR results are

normalized to *L19* expression. Mann-Whitney U test was utilized as statistical tests for the data presented in **A**, **B**, and **C**. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Fig. 3. A: High concentration of progesterone (P_4) and moderate levels of androstenedione (A-dione), testosterone (T), and dihydrotestosterone (DHT) are detected in the adrenal glands of intact and orchectomized mice. **B:** Adrenal to serum ratios for P_4, A-dione, T, and DHT, indicating their local production. **A** and **B:** Intact n=14 and ORX n=11. All measurements were performed at the end of the study, 63 days after ORX and 110 or with intact 84 days after cell inoculations. Data are expressed as median and range. Mann-Whitney U test was utilized for statistical analysis. $***P < 0.001$.

Fig. 4. A: Concentrations of serum progesterone (P_4), androstenedione (A-dione), testosterone (T), and dihydrotestosterone (DHT) in intact (n=15), orhectomized (ORX n=14), and ORX+ADX (n=13) mice. **B:** ORX alone has no effect on intratumoral P_4, T, or DHT, whereas adrenalectomy (ADX) combined with ORX results in a marked decrease in intratumoral steroids. **C:** Tumor to serum ratios for P_4, A-dione, T, and DHT indicate an intratumoral production of active androgens in ORX and ORX+ADX mice. **B** and **C:** Intact n=10, ORX n=14, and ORX+ADX n=13. All measurements were performed at the end of the study, about 62 days after ORX and ORX+ADX or with intact 84 days after cell inoculations. Data are expressed as median and range. Kruskal-Wallis with Dunn's post hoc test was utilized for statistical analyses. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Fig. 5. A: Expression of full-length AR (AR-FL), and AR-V1 and AR-V7 splice variant mRNA is increased in VCaP tumors by orchectomy (ORX), and the expression of the mRNAs show trend for further increase by adrenalectomy (ORX+ADX). **B:** Western blot analysis of AR protein in the VCaP tumors indicate that the induction of mRNA expression by ADX in ORX mice is not

translated to a higher AR protein expression, whereas ORX markedly increases both mRNA and protein levels. The intensities of AR signals are normalized to that of the beta-actin, and the values relative for that obtained for the tumors in intact mice are shown for the tumors in ORX and ORX+ADX mice. **C:** Expression of mRNAs for steroid synthetic enzymes measured by RT-qPCR, showing only modest differences between intact, ORX, and ADX+ORX groups. **D:** Relative mRNA expression of androgen-regulated genes *SYTL2*, *NOV*, *FKBP5*, ST6Ga1NAc1, *PMEPA1*, *KLK2*, *KLK*3, and *KLK4* in intact, ORX, and ORX+ADX tumors measured by RT-qPCR. All measurements were performed at the end of the study, about 63 days after ORX and ORX+ADX or with intact 84 days after cell inoculations. RT-qPCR results are normalized to *L19* expression. Data are expressed as mean±SEM (**A**, **C**, and **D**). The Kruskal-Wallis with Dunn's post hoc test was utilized for statistical testing of the data in **A**, **C**, and **D**. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

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Table 1: Real-Time Quantitative Reverse Transcription-PCR Primer Sequences

Human gene	Forward	Reverse
AKR1C2	5'- CCTAAAAGTAAAGCTCTAGAGGCCGT4 -3'	5'- GAAAATGAATAAGATAGAGGTCAACATAG -3'
AKR1C3	5'- GCCAGGTGAGGAACTTTCAC -3'	5'- CAATTTACTCCGGTTGAAATACG -3'
AR-FL	5'- CTTACACGTGGACGACCAGA -3'	5'- GCTGTACATCCGGGACTTGT -3'
AR-V1	5'- CCATCTTGTCGTCTTCGGAAATGTTATGAAGC -3'	5'- CTGTTGTGGATGAGCAGCTGAGAGTCT -3'
AR-V7	5'- CCATCTTGTCGTCTTCGGAAATGTTATGAAGC -3'	5'- TTTGAATGAGGCAAGTCAGCCTTTCT -3'
FKBP5	5'- AAAAGGCCACCTAGCTTTTTGC -3'	5'- CCCCCTGGTGAACCATAATACA -3'
HSD17B3	5'- CTGAAGCTCAACACCAAGGTCA -3'	5'- CTGCTCCTCTGGTCCTCTTCAG -3'
KLK2	5'- CTGCCCATTGCCTAAAGAAGAA -3'	5'- GGCTTTGATGCTTCAGAAGGCT -3'
KLK3	5'- CCAAGTTCATGCTGTGTGCT -3'	5'- GGTGTCCTTGATCCACTTCC -3'
KLK4	5'- GGCACTGGTCATGGAAAACGA -3'	5'- TCAAGACTGTGCAGGCCCAGC -3'
NOV	5'- ACCGTCAATGTGAGATGCTG -3'	5'- TCTTGAACTGCAGGTGGATG -3'
PMEPA1	5'- TGCCGTTCCATCCTGGTT -3'	5'- AGACAGTGACAAGGCTAGAGAAAGC -3'
L19	5'- AGGCACATGGGCATAGGTAA -3'	5'- CCATGAGAATCCGCTTGTTT -3'
SRD5A1	5'- CCTGTTGAATGCTTCATGACTTG -3'	5'- TAAGGCAAAGCAATGCCAGATG -3'
SRD5A2	5'- CTCTCTAAGGAAGGGGCCGAAC -3'	5'- GACAATGCATTCCGCAAACATA -3'
ST6GalNAc1	5'- AGGCACAGACCCCAGGAAG -3'	5'- TGAAGCCATAAGCACTCACC -3'
SYTL2	5'- TCTGCCTTGAGAAAACAAACAGTT -3'	5'- GCCAGTGGGTGGCACTAAAA -3'
Mouse gene	**Forward**	**Reverse**
Ar	5'- GTCTCCGGAAATGTTATGAA -3'	5'- AAGCTGCCTCTCTCCAAG -3'
Akr1c6	5'- CAGACAGTGCGTCTAAGTGATG -3'	5'- CGGATGGCTAGTCCTACTTCCT-3'
Akr1c18	5'- TGGCACTGTGAAAAGGGAAGAT -3'	5'- TTAGGCAAAAGCTCATTCCCTGG -3'
Akr1d1	5'- TTGCGTTTCAACATCCAGCG -3'	5'- AGCAACTCCACATAGCGGAC -3'
Cyp11a1	5'- AGATCCCTTCCCCTGGCGACAATG -3'	5'- CGCATGAGAAGAGTATCGACGCATC -3'
Cyp17a1	5'- CAAGCCAAGATGAATGCAGA -3'	5'- AGGATTGTGCACCAGGAAAG -3'
Hsd3b1	5'- CAGGAGCAGGAGGGTTTGTG -3'	5'- GTGGCCATTCAGGACGAT -3'
Hsd3b2	5'- CAGTTGTTGGTGCAAGAGGA -3'	5'- CCTGGGAATGACACCTGTGA -3'
Hsd17b3	5'- CACGGGGATAAAGACCAGGT -3'	5'- GATCGCAGGAAAGAGCTTGG -3'

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Hsd17b6	5'- TTTGGAGGATTCTACAGTTGCTC -3'	5'- TCACCCCGAAATCTTGAACCT -3'
L19	5'- GGACAGAGTCTTGATGATCTC -3'	5'- CTGAAGGTCAAAGGGAATGTG -3'
Lhcgr	5'- GCCCTGAGCCCTGCGACTGC -3'	5'- AAAGCGTTCCCTGGTATGGTGGTT -3'
Mc2r	5'- TCTGACATCATGTTGGGCAGTCT -3'	5'- TGGTGATGTAACGGTCAGCT -3'
Srd5a1	5'- TGAGCCAGTTTGCGGTGTAT -3'	5'- CTCCACGAGCTCCCCAAAAT -3'
Srd5a2	5'- CACAGACATGCGGTTTAGCG -3'	5'- AACAAGCCACCTTGTGGGAT -3'
Srd5a3	5'- CTGGCTTAGTGCTCTGCTCA -3'	5'- CACAACGTGAATGGCTGCAT -3'
Ugt1a1	5'- GCAGAGTGGTTTATTCCCCCT -3'	5'- AGGCGTTGACATAGGCTTCAA -3'

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Figure 1

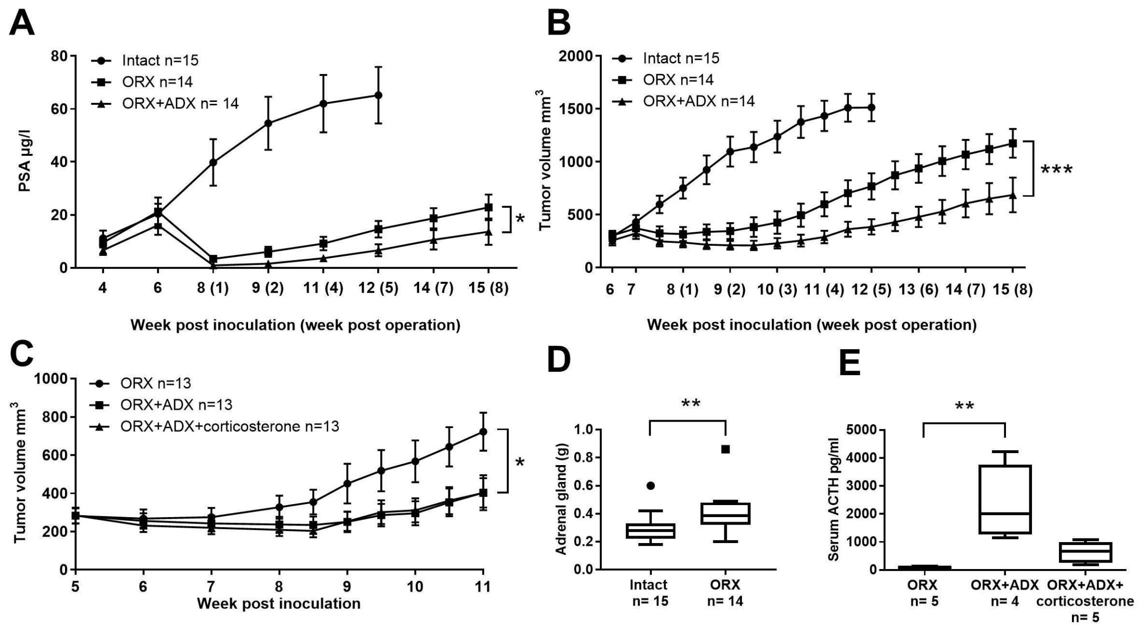

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Figure 2

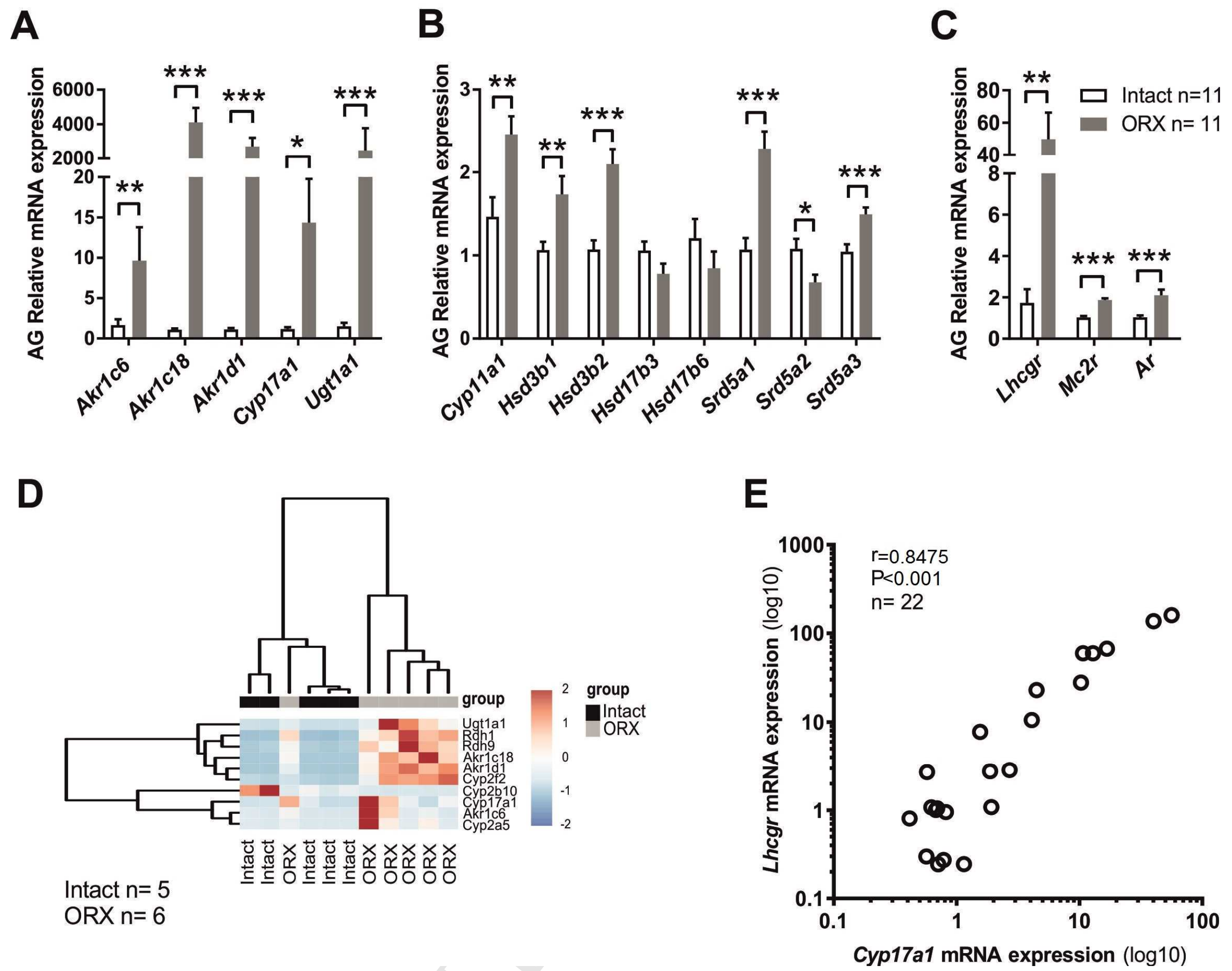

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Figure 3

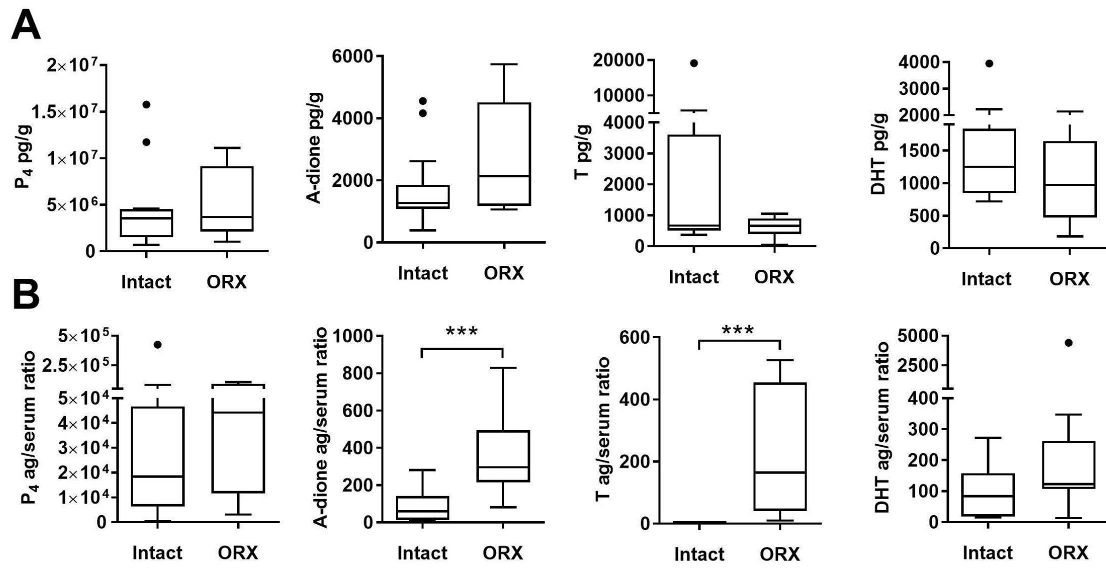

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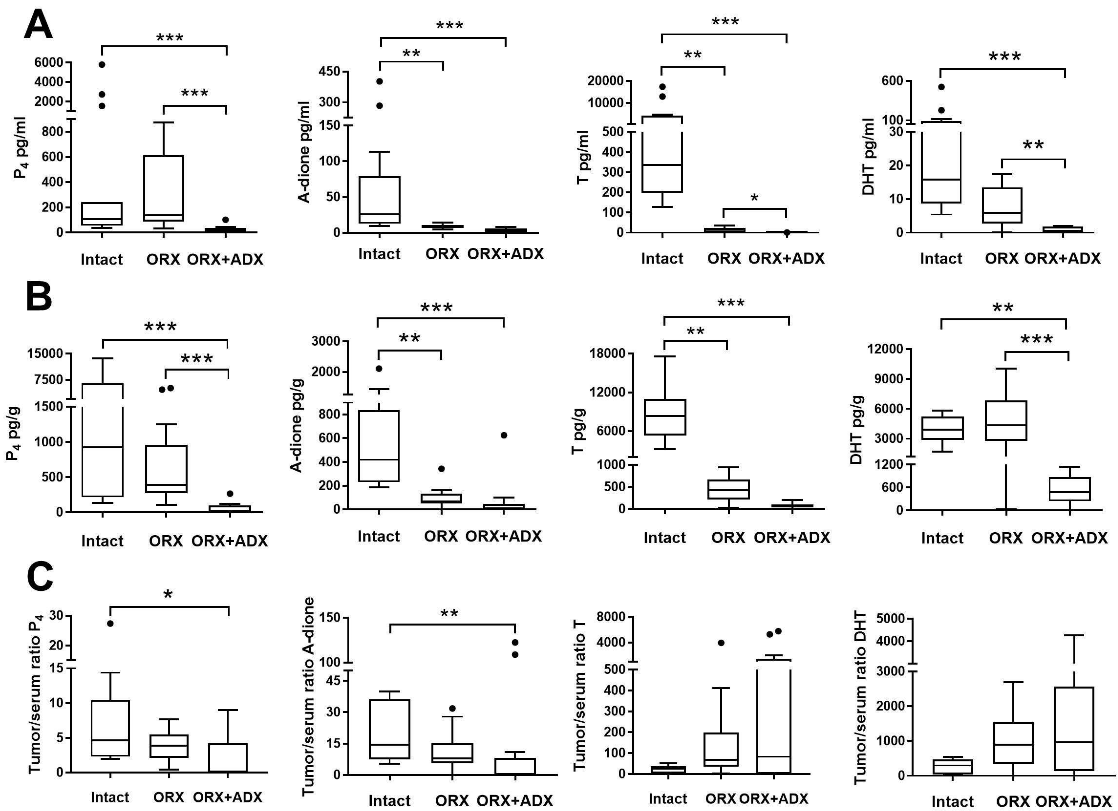
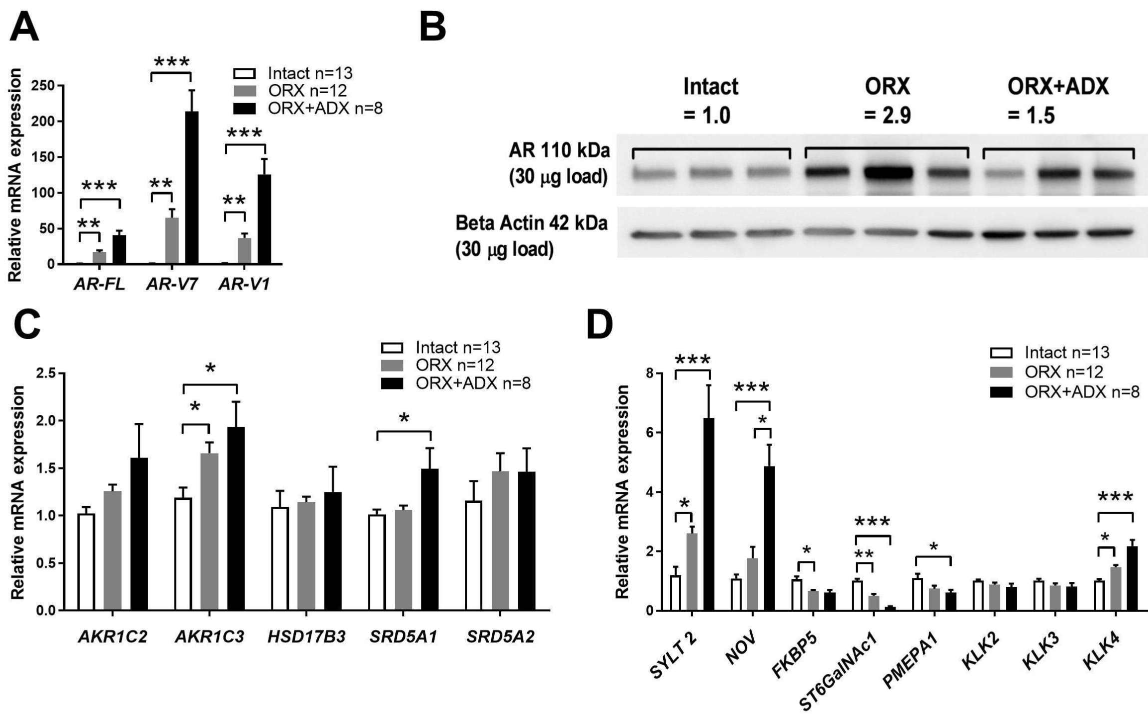
Figure 4





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