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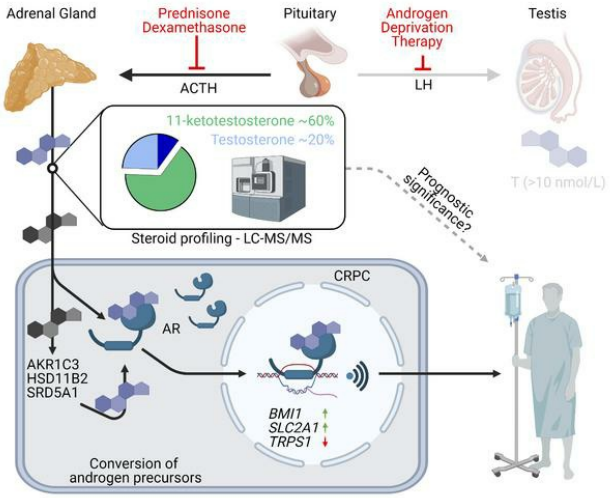
Graphical abstract



11-Ketotestosterone: the resilience of a potent androgen in prostate cancer patients after castration

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Abbreviations:

General

ADT	Androgen deprivation therapy
AR	Androgen receptor
CRPC	Castration-resistant prostate cancer
HPA	Hypothalamus-pituitary-adrenal
IQR	Interquartile range
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LOQ	Limit of quantification
OT	On treatment
PC	Prostate Cancer
PD	Progressive Disease
PFS	Progression-free survival
TA	Total active androgens

Steroids

11KA4	11-ketoandrostenedione
11KT	11-ketotestosterone
11OHA4	11β-hydroxyandrostenedione
11OHT	11β-hydroxytestosterone
DHT	Dihydrotestosterone
T	Testosterone

ABSTRACT (242/250)

Background

Continued androgen receptor (AR) signaling constitutes a key target for treatment in metastatic castration-resistant prostate cancer (CRPC). Studies have identified 11-ketotestosterone (11KT) as a potent AR agonist, but it is unknown if 11KT is present at physiologically-relevant concentrations in CRPC patients to drive AR activation. The goal of this study was to investigate the circulating steroid metabolome including all active androgens in CRPC patients.

Methods

Metastatic CRPC patients (n=29) starting a new line of systemic therapy were included. Sequential plasma samples were obtained for measurement of circulating steroid concentrations by multi-steroid profiling employing liquid chromatography-tandem mass spectrometry. Metastatic tumor biopsy samples were obtained at baseline and subjected to RNA sequencing.

Results

11KT was the most abundant circulating active androgen in 97% of CRPC patients (median 0.39 nmol/L, range: 0.03–2.39 nmol/L), constituting 60% (IQR 43-79%) of the total active androgen (TA) pool. Treatment with glucocorticoids reduced 11KT by 84% (49-89%) and testosterone (T) by 68% (38-79%). Circulating TA concentrations at baseline were associated with a distinct intratumoral gene expression signature comprising AR-regulated genes.

Conclusions

The potent AR agonist 11KT is the predominant circulating active androgen in CRPC patients and, therefore, one of the potential drivers of AR activation in CRPC. Assessment of androgen status should be extended to include 11KT, as current clinical approaches likely underestimate androgen abundance in CRPC patients.

Trial registration

Netherlands Trial Register: NL5625(NTR5732)

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INTRODUCTION

Targeting the androgen receptor (AR) pathway through androgen deprivation therapy (ADT) is the mainstay of treatment in metastatic prostate cancer (PC) (1). Eventually, most tumors will evolve from hormone-sensitive to castration-resistant prostate cancer (CRPC) and show progression despite suppressed testosterone (T) levels. The continued importance of the AR pathway in tumor growth and progression has been underlined by the efficacy of novel drugs targeting the AR pathway (2-5). AR upregulation (6), increased intratumoral conversion of adrenal androgen precursors (7-9), non-canonical dihydrotestosterone (DHT) biosynthesis (10), and downregulation of androgen inactivating enzymes (11, 12) may all contribute to AR pathway reactivation.

In recent years, novel androgenic steroids have been identified with significant AR activation potential (13). The adrenal-derived steroid 11-ketotestosterone (11KT) is of particular interest, as it is one of a few endogenous steroids capable of activating the AR at sub-nanomolar concentrations, similar to T and DHT (13-15). In healthy adult men, circulating T concentrations exceed those of 11KT (16, 17). During ADT, gonadal steroidogenesis is inhibited and T concentrations typically fall below 0.5 nmol/L (18), which is lower than 11KT concentrations in healthy men (16).

We hypothesized that due to the adrenal origin of its precursors, 11KT may persist after castration and may therefore exceed the residual concentrations of T and DHT in CRPC patients. Similarly, the 11-oxygenated precursor steroids 11β-hydroxyandrostenedione (11OHA4), 11-ketoandrostenedione (11KA4) and 11β-hydroxytestosterone (11OHT), which can be converted

112 into 11KT (**Figure 1**), may persist after castration and serve as substrate for intratumoral
113 androgen production. Androgen abundance after castration has been associated with improved
114 response to AR pathway inhibition in CRPC patients(19-21). Thus, persistence of a previously
115 overlooked, potent androgen class would be of major clinical significance in CRPC patients.

116 In this study, we carried out mass spectrometry-based multi-steroid profiling in the plasma of
117 29 patients before, during and after treatment for CRPC. We report the abundance of
118 circulating active androgens in these patients. Additionally, we show the effects imposed on the
119 steroid metabolome by treatment with exogenous glucocorticoids. Finally, we show that total
120 androgen levels including 11KT are linked to differential gene expression in tumor biopsies.

121

RESULTS

Patients

Samples used in this study were obtained between May 2016 and July 2018 from 30 patients with metastatic CRPC who were scheduled to start a new line of treatment. One patient was excluded based on non-castrate T concentrations at baseline. In total, 29 patients who completed 34 treatments were included in our analysis (**Figure 2**); five patients completed two treatments during their enrollment. Patient characteristics, disease and treatment history are shown in **Table 1**. For five patients with early progression (progression-free survival (PFS): 22–82 days) no separate on treatment (OT) sample was available. These subjects were included in the comparison between baseline and OT samples, but not between OT and progressive disease (PD) samples.

Androgen Abundance

The circulating active androgen concentrations in CRPC patients were determined by liquid-chromatography-tandem mass spectrometry (LC-MS/MS) in samples obtained before the start of the first treatment after enrollment (n=29). The median concentration of 11KT (0.33 nmol/L, range 0.03–2.39 nmol/L) was significantly higher than T (0.12 nmol/L; range 0.03–0.64 nmol/L, $p<0.001$) in CRPC patients at baseline (**Figure 3A**).

11KT constituted 60% (43–79%) of the total active androgen (TA) pool whereas T was 20% (15–32%) (**Figure 3B**). Although T was below the castrate cutoff (1.74 nmol/L, 50 ng/mL) in all baseline samples (n=34), the TA concentration (0.59 nmol/L, 0.23-1.27 nmol/L) exceeded 1.74

nmol/L in six patients (**Figure 3D**). DHT was below the lower limits of quantification (LOQ) in most patients (range: <LLOQ–0.27 nmol/L). The correlation between most of the 11-oxygenated androgens was high (R^2= 0.71 – 0.87, respectively, **Supplemental Figure S1**), with the exception of 11OHT (R^2= 0.39 – 0.55). Of the 11-oxygenated precursor steroids, 11OHA4 showed the closest correlation with testosterone (R^2 = 0.73), 11KT (R^2= 0.87) and TA (R^2= 0.86).

Effects of Treatment

Subjects included in this study started treatment with AR antagonists (n=10), docetaxel with prednisone (n=10) or cabazitaxel with prednisone (n=14). Steroid hormone concentrations at baseline stratified for the different treatments are shown in **Supplemental Figure S2A-D**. Significant suppression of adrenal-derived steroids was observed after 12 weeks of cabazitaxel with prednisone treatment. In the docetaxel group, a similar suppression was observed in a subset of patients, but overall, this suppressive effect did not reach significance. In the AR antagonist group, increased steroid concentrations were observed after 12 weeks of treatment.

Low baseline cortisol concentrations were detected in a subset of patients, suggestive of hypothalamus-pituitary-adrenal (HPA) axis suppression by exogenous glucocorticoids. Post-hoc exogenous glucocorticoid quantification by LC-MS/MS was performed to detect prednisone, prednisolone and dexamethasone in all samples (**Supplemental Figures S3A-C**). Samples were classified as exogenous glucocorticoid positive if prednisolone (≥20.7 ng/mL) and/or dexamethasone (≥16.1 ng/mL) were detected. Cortisol was suppressed (<140 nmol/L) in all exogenous glucocorticoid positive baseline samples. (**Supplemental Figure S3D).**

A significant reduction in circulating glucocorticoids as well as T and 11KT concentrations was observed in patients treated with exogenous glucocorticoids (**Figure 4A**). Circulating T (**Figure 4B**) and 11KT (**Figure 4C**) concentrations were lowered by 68% (interquartile range (IQR): 38–79%) and 84% (49–89%), respectively, in patients starting exogenous glucocorticoids. Decreases of similar magnitude were observed for 11OHA4, 11OHT and 11KA4 (medians 66-92%; **Figure 4A**). In a subset of exogenous glucocorticoid-treated patients, glucocorticoid treatment was withdrawn. The group size was insufficient to detect a statistical difference between baseline and treatment (n=5, **Figure 4D**). However, compared to patients who continued exogenous glucocorticoid treatment (n=10), withdrawn patients had eight-fold higher T (0.30 nmol/L (0.26–0.73 nmol/L) vs 0.04 nmol/L (0.02–0.05 nmol/L)) and ten-fold higher 11KT (1.09 nmol/L (0.75-2.30 nmol/L) vs 0.11 nmol/L (0.04–0.23 nmol/L)) (**Figures 4E and 4F**). An overview of steroid concentrations at baseline can be found in **Supplemental Table T1**. Additionally, exogenous glucocorticoid treatment was withdrawn in six patients before progression. Again, higher median circulating concentrations of T (0.20 nmol/L (0.09–0.38 nmol/L) vs 0.05 nmol/L (0.02–0.08 nmol/L), $p<0.01$) and higher median 11KT (0.90 nmol/L (0.52–1.46 nmol/L) vs 0.10 nmol/L (0.06–0.29 nmol/L), $p=0.001$) were observed after withdrawal, compared to patients who continued glucocorticoid treatment (n=14, **Supplemental Figure S4**).

Clinical outcomes

A post-hoc survival analysis was performed on this limited patient group. High TA concentrations (above the median) associated with a longer PFS in patients (209 vs 133.5 days, $p<0.05$, **Figure 5A**). Stratification based on 11KT alone similarly showed this association (**Figure**

5B), whereas T alone did not (**Figure 5C**). None of the 11-oxygenated androgen precursors were independently associated with survival (**Supplemental Figure S5**). Overall survival was not affected by TA pool quantities (14.7 months vs 12.3 months, P>0.05).

RNA sequencing analysis

Gene expression profile analysis of the complete CPCT-02 CRPC cohort (n=180) revealed significant biopsy-site related bias, which we attempted to limit through exclusion of 5232 biopsy-site specific genes (**Supplemental Figure S6**). Next, biopsy material obtained from 15 of the patients included in this study were assessed by RNA sequencing, excluding genes which were biopsy-site related **Supplemental Figure S7**). Using TA concentration as a continuous variable, we observed androgen-mediated differential expression of 24 genes (**Figure 5D**), including several known androgen-regulated genes. Of those, 12 were upregulated in the high TA environment, including the known AR target genes *BMI1(22)* and *SLC2A1(23)*, and the other 12 in the low TA environment, including the androgen-repressed gene *TRPS1(24)*. Furthermore, a trend towards increased *AR* expression in the low TA environment (5.4-fold higher) was observed, although this did not reach statistical significance.

Several enzymes related to androgen biosynthesis and (re-)activation were highly expressed in all tumor biopsy samples, including *HSD17B10*, *STS*, *SRD5A1*, *AKR1C3* and *HSD11B2* (**Supplemental Figure S8**). This suggests that the enzymes required for activation of both androgen and 11-oxygenated androgen precursors are expressed in CRPC tumors.

DISCUSSION

Using mass spectrometry-based plasma multi-steroid profiling, we show that the potent AR agonist 11KT is the predominant circulating active androgen in CRPC patients and therefore needs to be considered when assessing the hormonal status of these patients. 11KT constituted a median 60% of the active androgen pool, signifying that androgen abundance in CRPC patients is currently systematically underestimated. Estimation of the TA abundance after castration could be assessed more accurately by including 11KT. Additionally, this study shows the suppressive effects of exogenous glucocorticoids on circulating androgen concentrations, highlighting the potential therapeutic role of glucocorticoids in CRPC, which crucially includes suppression of adrenal-derived 11-oxygenated androgens.

AR pathway reactivation after ADT is an important process that leads to tumor progression. In most patients, 11KT was more abundant than T and DHT combined, and our data suggest that 11KT may be an important contributor to AR reactivation in CRPC. In addition to direct activation of the AR by active androgens, intratumoral conversion of precursor steroids into active androgens may further contribute to AR reactivation in. Storbeck *et al.* (25) previously showed that the PC cell line LNCaP converts 11OHA4 and 11OHT into 11KT, requiring the enzymes HSD11B2 and AKR1C3. We confirm substantial expression of *HSD11B2* and *AKR1C3* in nearly all CRPC tumor samples (93% and 100%, respectively). Expression of enzymes that inactivate (11-oxygenated) androgens to their upstream precursors, such as *HSD17B2* and *HSD11B1,* was lower. A higher ARK1C3:HSD17B2 ratio favors production of 11KT especially, as AKR1C3 has a significantly higher substrate preference for 11KA4 than androstenedione(26).

The intratumoral androgen levels could not be studied in this study, however, and this is necessary to fully elucidate the role of the 11-oxygenated androgen precursors.

We found that the 11-oxygenated androgen precursors 11OHA4 and 11KA4 persist after castration and correlate with 11KT. The concentrations detected in our patients without exogenous glucocorticoid treatment are comparable to previously reported concentration of 11OHA4 and 11KA4 in elderly men (17). In line with previous reports, we found that 11OHA4 is the most abundant 11-oxygenated androgen in the circulation, with a median concentration of 2.7 nmol/L (0.48 – 5.1 nmol/L) in all CRPC patients, and 4.96 nmol/L (3.05 – 6.13 nmol/) in patients who did not receive exogenous glucocorticoid treatment. 11OHT was the least abundant of the 11-oxygenated androgens studied. Due to the low concentrations and relatively lower affinity for the AR (25), it was not included in our calculation of the TA pool. Androstenedione could not be quantified in our study due to a technical limitation, but the concentration in CRPC patients has been determined in earlier studies (18).

Glucocorticoid treatment significantly decreased circulating T and 11-oxygenated androgens through suppression of the HPA-axis. Potent suppression of 11-oxygenated androgens has previously been observed in patients treated with abiraterone acetate and prednisone (27). Abiraterone acetate suppresses the production of adrenal androgens through direct inhibition of CYP17A1, rather than negative feedback. Prednisone is administered to these patients to prevent increased production of adrenocorticotropic hormone (ACTH), which may otherwise lead to mineralocorticoid excess. The potent suppression of adrenal androgens, and 11KT in particular, may partially explain the clinical benefits of glucocorticoid treatment independent of

abiraterone and should be considered when designing trials with glucocorticoid treatment in the control arm(28, 29). Withdrawal of glucocorticoid treatment may inadvertently lead to an increase in circulating androgens, although the effect on CRPC tumor growth is still unknown.

Gene expression analysis of the tumor biopsy samples in a subset of patients identified 24 genes differentially expressed between patients across the TA spectrum, including two androgen-stimulated genes and one androgen-repressed gene (22-24). *TRPS1* and *SLC2A1* have previously been implicated in PC and AR action (30, 31), and decreased *EFS* expression has been associated with more advanced PC and tumor recurrence (32, 33). Other genes differentially expressed in our cohort (*EDA2R, SLC17A9*, *TDRD10*, *ALDOC*, *SRRM3*, *MEST* and *RTKN2*) have been implicated in other malignancies, but not in PC specifically (34-38).

PFS was longer in our patients with high TA, in line with earlier findings that higher T concentrations at the CRPC stage are associated with a more favorable response to AR pathway inhibition (19-21). Interestingly, in our study this association was attributed to 11KT, as T alone was not associated with PFS. In another study, improved outcome in docetaxel with prednisone treated CRPC patients was associated with androstenedione (39). Although androstenedione could not be quantified in this study, it has been previously shown that androstenedione levels correlate with the levels of 11OHA4 and 11KT (40). Together, these findings indicate that the adrenal androgens and precursor steroids should be further investigated as potential prognostic markers. We hypothesize that while adrenal androgens persist, cancer cells that rely on intratumoral conversion of precursors and ligand-dependent AR activation may have a competitive advantage, resulting in a selection for cells that inadvertently remain responsive to

conventional treatments that target the AR signaling pathway. However, AR pathway inhibition may result in the development of androgen-independent resistance mechanisms, such as expression of AR variants (41), the glucocorticoid receptor (42), or development of a neuroendocrine phenotype (43), which is associated with poor prognosis (44, 45). CRPC cells in lower androgen milieu may be under increased selective pressure for adverse disease characteristics, contributing to lower PFS. It is, however, important to consider that our study was not designed to detect differences in survival, and the limited sample size and patient heterogeneity do not permit a definitive conclusion in this regard. Most patients in the CIRCUS study had previously received treatment for CRPC. No association between the number of treatment lines and PFS was found, nor was the number of treatment lines different between the low and high TA groups. Further investigation into the actions and consequences of circulating 11KT is warranted, especially as a potential biomarker to select patients more likely to respond to AR-targeting therapies.

Conclusion

This study demonstrates that 11KT is the predominant circulating active androgen in CRPC patients. Paired with evidence from previously published findings, our results position 11KT as one of the potential drivers of AR activation in CRPC. Both T and 11KT can be suppressed by glucocorticoid treatment, providing a possible explanation why glucocorticoids are beneficial in CRPC patients. Future studies should consider the total active androgen pool as a potential biomarker in patients who have undergone ADT.

METHODS

Patients and Samples

From April 2016 onwards, metastatic CRPC patients who continued ADT and intended to start a new line of systemic therapy were included in the *CIRCUS* study (Netherlands Trial Registry ID: NL5625). Metastatic disease and progression were defined according to the PCWG2 and/or RECIST 1.1 criteria (46, 47). All patients who started treatment with AR antagonists (enzalutamide or apalutamide), docetaxel with prednisone or cabazitaxel with prednisone were included in our analysis if blood samples were available at both baseline and upon progression (**Figure 2**). Concurrent participation in the CPCT-02 study (NCT01855477) was required to obtain a tumor biopsy before start of therapy. Blood was collected in three CellSave preservative tubes (Menarini Sillicon Biosystems Inc, Huntington Valley, PA, USA) every three to four weeks, including at baseline, OT and at PD. Plasma was isolated and stored as previously described (48, 49).

Measurement of circulating steroids

Calibration series (0.01–100 ng/mL) were prepared in duplicate in phosphate-buffered saline with 0.1% bovine serum albumin and in charcoal-stripped human serum (Goldenwest Diagnostics, Temecula, CA, USA). Extraction and quantification of steroids was performed as previously described(40, 49-52). Briefly, steroids were obtained from 400 µL plasma by liquid-liquid extraction. Multi-steroid profiling was performed by LC-MS/MS (Xevo TQ-XS, Waters, Milford, MA, USA) after separation on an ACQUITY UPLC (Waters) with UPLC HSS T3 column (21 mm x 50 mm, 1.8 µm, Waters). A representative chromatogram is shown in **Supplemental**

Figure 9 and an overview of included steroids, their mass transitions, internal standards and LOQ can be found in **Supplemental Table T2.** The **Supplementary Methods** section contains additional information regarding extraction, accuracy, precision and values below LOQ. Masslynx (v4.1, Waters) was used to process LC-MS/MS data. The TA pool was defined as the sum of T, DHT and 11KT concentrations, as these steroids exert strong agonistic activity at the androgen receptor (13).

RNA sequencing

As part of the CPCT-02 study (NCT01855477) metastatic CRPC biopsy samples of patients included in this analysis were obtained at baseline. RNA sequencing was performed according to the manufacturer protocols using a minimum of 100 ng total RNA input. Total RNA was extracted using the QIAGEN QIAsymphony kit (Qiagen, FRITSCH GmbH, Idar-Oberstein, Germany). Paired-end sequencing of (m)RNA was performed on the Illumina NextSeq 550 platform (2x75bp; Illumina) and NovaSeq 6000 platform (2x150bp; Illumina). Downstream data processing and analysis is detailed in the **Supplemental Methods**. Briefly, CRPC tumor biopsies from the entire CPCT-02 study (n=180) were used to identify biopsy-site specific genes. Subsequently, an untargeted approach was used to analyze gene expression across TA concentrations in biopsy samples of patients included in this study only (n=15), excluding biopsy-site related genes. The expression of steroid hormone receptors and genes involved in steroid metabolism was assessed using a targeted approach(53).

Statistical analysis

Statistical analyses were performed with Graphpad Prism (version 6.01, La Jolla, California, USA), SPSS (version 26, IBM Corp., Armonk, New York, USA) and R (version 3.6.1, Vienna, Austria). Logarithmic transformation was applied if obtained steroid concentrations did not pass D'Agostino and Pearson's test for normality. We performed one-way ANOVA with post-hoc Dunnett test to compare circulating androgen concentrations. Wilcoxon's signed-rank test was performed to assess the effects of treatment. Mann-Whitney tests were used to compare the difference between exogenous glucocorticoid treated and untreated patients after 12 weeks of treatment. Linear models were used to assess how the individual active androgens were associated with TA. The associations between PFS and androgens were investigated at baseline and during treatment by the Kaplan-Meier method and differences compared by Log-rank test. Correlation (Pearson) between androgens was determined after logarithmic transformation of baseline values. Group steroid concentrations and changes were reported as median with IQR, unless stated otherwise. A P-value less than 0.05 was considered significant.

Study Approval

The *CIRCUS* study (Netherlands Trial Registry ID: NL5625) was approved by the medical ethics board of the Erasmus Medical Center (MEC-2016-081). All patients provided written informed consent before any study procedure; this involved blood collection at baseline, OT, at PD, and collection of clinical data.

Author Contributions

These authors contributed equally: Gido Snaterse and Lisanne F van Dessel. Gido Snaterse was selected as the first of the two authors as he was responsible for the LC-MS/MS experiments. Dr Hofland had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis

Study concept and design: De Wit, Visser, Arlt, Lolkema, Hofland

Patient recruitment: van Dessel, Hamberg, De Wit, Lolkema

Acquisition, analysis or interpretation of data: All authors

Drafting of the manuscript: Snaterse, van Dessel, van Riet

Critical revision of the manuscript: All authors

Statistical analysis: Snaterse, van Dessel, van Riet

Obtained funding: Hofland, Arlt

Administrative, technical or material support: Taylor, van der Vlugt-Daane

Study supervision: Visser, Lolkema, Hofland

All authors reviewed and approved the final version of the manuscript

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Data Availability

The LC-MS/MS datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request. The presented whole-transcriptome sequencing data (.fastq) and corresponding attributes have been requested from Hartwig Medical Foundation and were provided under data request number DR-071 (Feb. 2020). Both whole-transcriptome sequencing and clinical data is freely available for academic use from the Hartwig Medical Foundation through standardized procedures and request forms can be found at https://www.hartwigmedicalfoundation.nl (54).

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Figure 1 – Androgen biosynthesis

Schematic overview of the conversion of adrenal precursor steroids to the potent androgens T, DHT and 11KT. The molecular structures of the active androgens are shown, with the 11-keto group highlighted in gray.

Abbreviations: DHEA – dehydroepiandrosterone

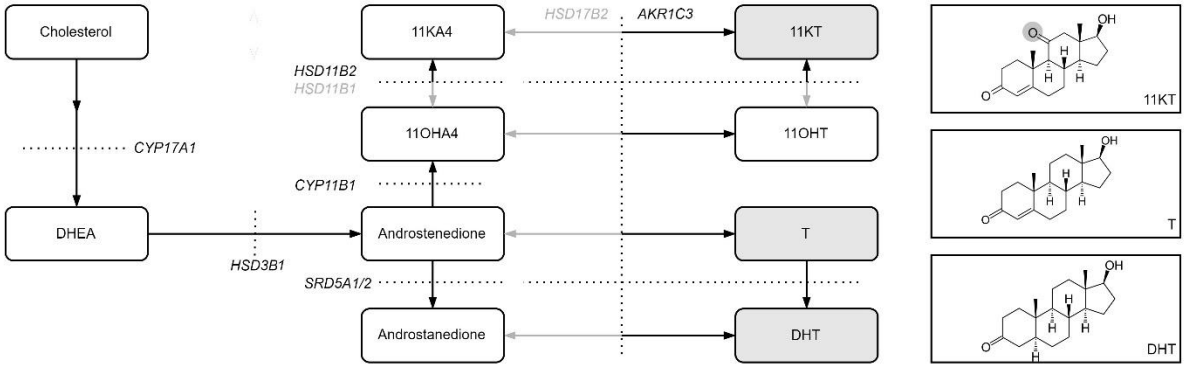


Figure 2 – Patient and sample selection.

Selection and exclusion of CIRCUS study samples for multi-steroid profiling, glucocorticoid quantification, survival analysis and tumor biopsy analysis.

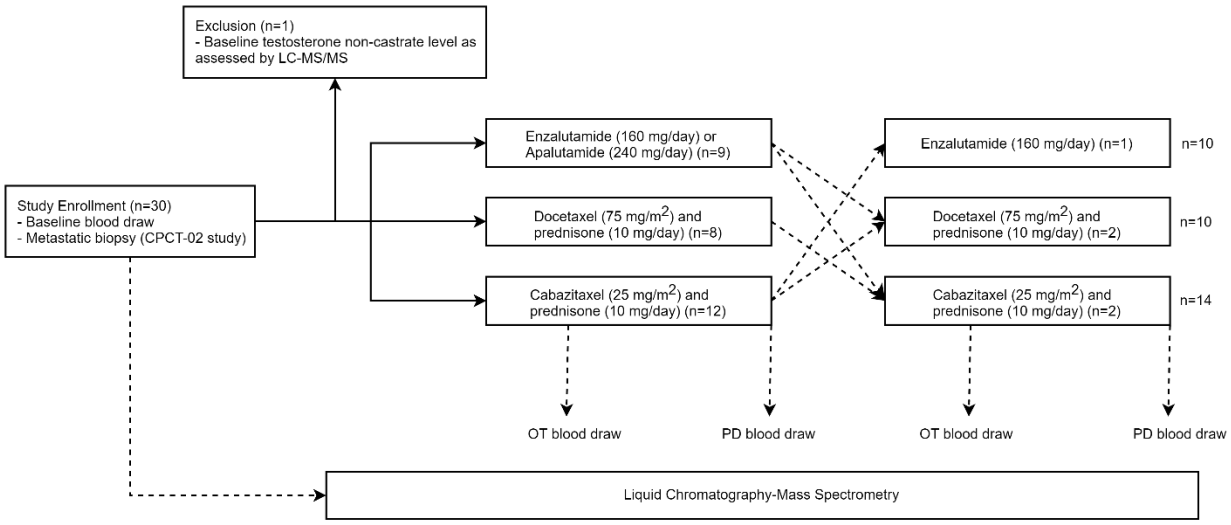
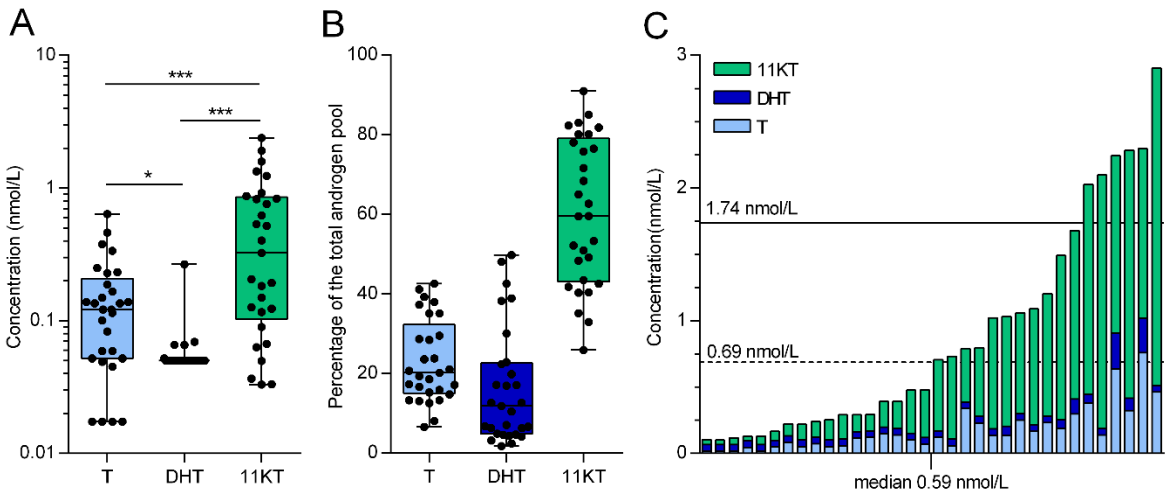


Figure 3 – 11-ketotestosterone is the most abundant circulating active androgen in castration-resistant prostate cancer patients at baseline. (**A**) Active androgen concentrations of all castration-resistant prostate cancer patients before the start of the first treatment after enrollment (n=29). Boxplot depicts the upper and lower quartiles, with the median shown as a solid line; whiskers indicate the range. Dots indicate individual data points. Statistical analysis was performed by one-way ANOVA ($p<0.0001$) with Tukey's Multiple Comparison test. * $p<0.05$, *** $p<0.001$. (**B**) The relative abundance of each androgen is shown as a percentage of the total androgen pool. Boxplot depicts the upper and lower quartiles, with the median shown as a solid line; whiskers indicate the range. Dots indicate individual data points. (**C**) Active androgen concentrations are shown for all baseline samples (n=34). Values below the analytical limit of quantification are shown if relevant calibrator and spiked quality control samples were accurate and reproducible with signal to noise >10:1. Samples with undetectable concentrations were set to 0.5 times the lowest accurate calibration sample for statistical purposes. Conventional clinical cut-off values for castrate testosterone levels (0.69 and 1.74 nmol/L (or 20 and 50 ng/dL) testosterone) are indicated on the y-axis for reference.


A
B
C
Concentration (nmol/L)
10
1
0.1
0.01
***
***
*
T
DHT
11KT
Percentage of the total androgen pool
100
80
60
40
20
0
T
DHT
11KT
Concentration(nmol/L)
3
2
1
0
11KT
DHT
T
1.74 nmol/L
0.69 nmol/L
median 0.59 nmol/L

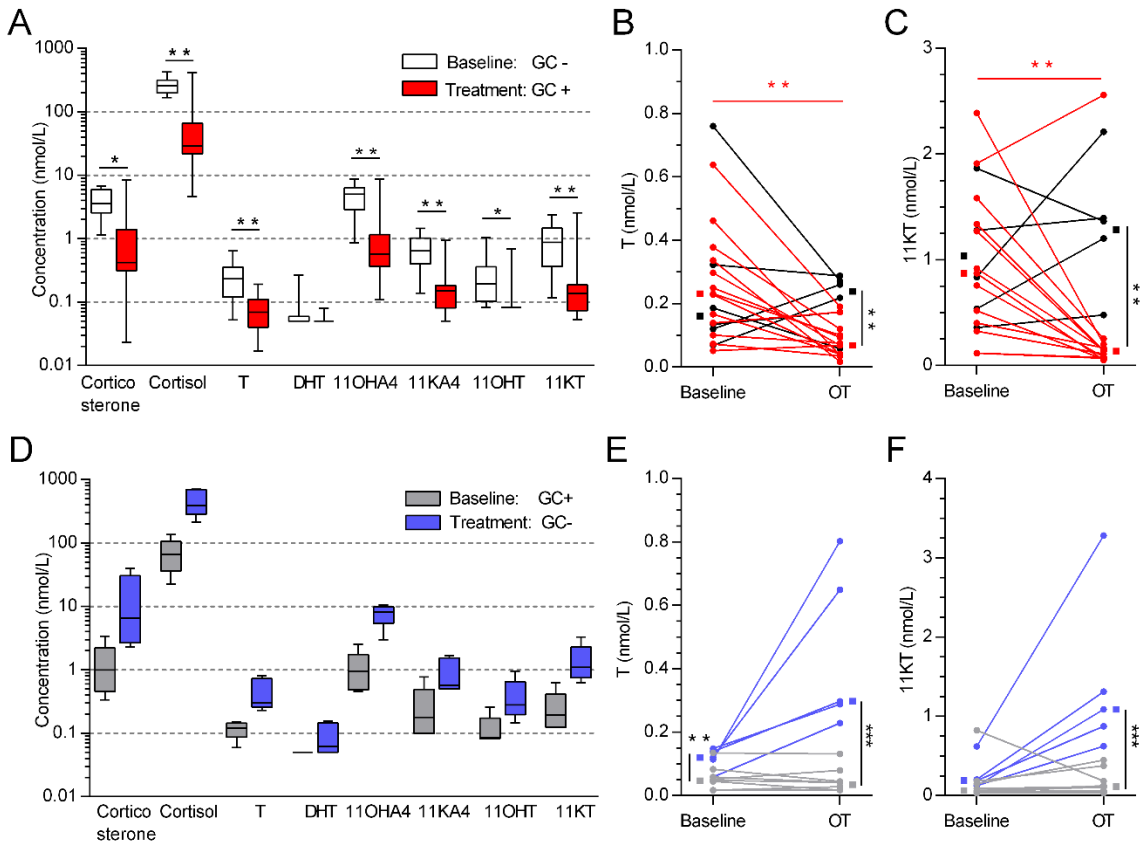
531

532

Figure 4. Effects of exogenous glucocorticoid treatment on circulating steroid concentrations. **(A)** Differences between steroid concentrations at baseline (white boxes) and OT (red boxes) were assessed in patients who were exogenous glucocorticoid untreated at baseline and who started treatment with glucocorticoids (n=13) by Wilcoxon signed-rank test. The individual data points are shown for (**B**) T and (**C**) 11KT at baseline and OT in patients who started therapy with glucocorticoids (red lines, n=13) or without glucocorticoids (black lines, n=6). (**D**) Differences between concentrations at baseline (gray boxes) and OT (blue boxes) were assessed in patients who were glucocorticoid treated at baseline and discontinued glucocorticoid treatment (n=5). The individual data points are shown for (**E**) T and (**F**) 11KT at baseline and OT in patients who continued treatment with glucocorticoids (gray lines, n=10) or were withdrawn from glucocorticoids (blue lines, n=5).

Boxplot depicts the upper and lower quartiles, with the median shown as a solid line; whiskers indicate the range. Effects of treatment were assessed by Wilcoxon ranked-sum test, while group differences were assessed by Mann-Whitney test. Lines connect individual patients and group medians (squares) are shown beside the individual data points. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Abbreviations: GC –glucocorticoids


A
B
C
D
E
F
Baseline: GC -
Treatment: GC +
Baseline: GC+
Treatment: GC-
Concentration (nmol/L)
Cortico sterone
Cortisol
T
DHT
11OHA4
11KA4
11OHT
11KT
T (nmol/L)
11KT (nmol/L)
Baseline
OT

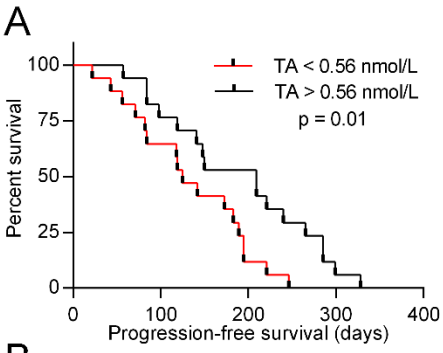
551

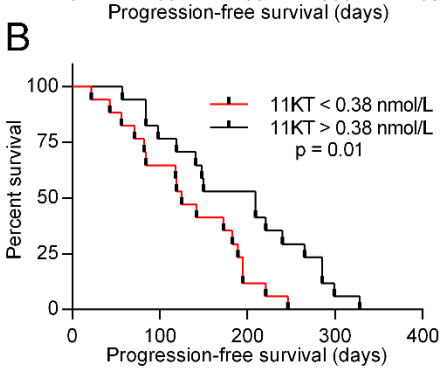
Figure 5 – Effects of total active androgen concentration on progression-free survival and intratumoral gene expression.

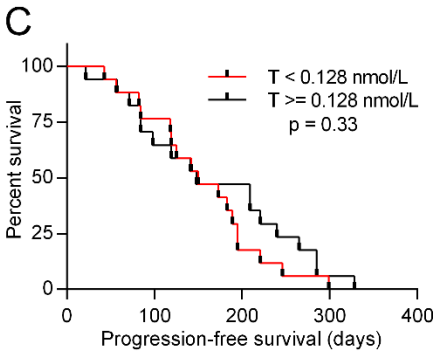
Progression-free survival curves are shown for patients stratified into two groups with concentrations above or below median (**A)** total active androgen (TA, defined as the sum of T, DHT and 11KT), (**B**) 11KT or (**C**) T. Log-rank test for survival was used to determine difference between the low and high TA groups.

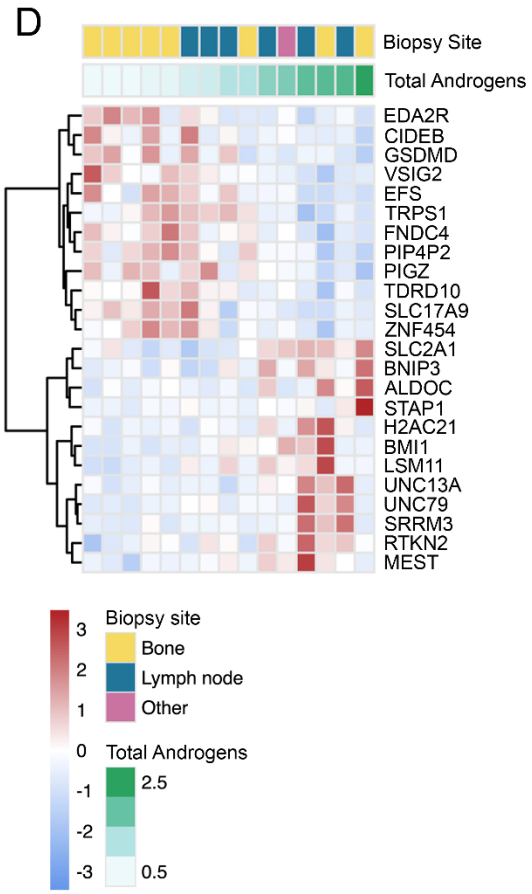
(**D**) Heatmap of differentially expressed genes (n=24) across TA concentration in the tumor samples. Differential gene expression was determined using TA concentration as a continuous variable. Heatmap displays mean-centered and normalized (variance-stabilizing transformation) read counts. Unsupervised hierarchical clustering (Euclidean distance and Ward.D2 method) was performed on genes and samples. Upper tracks display biopsy site and TA concentrations.

563


A
TA < 0.56 nmol/L
TA > 0.56 nmol/L
p = 0.01
Percent survival
Progression-free survival (days)


B
11KT < 0.38 nmol/L
11KT > 0.38 nmol/L
p = 0.01
Percent survival
Progression-free survival (days)


C
T < 0.128 nmol/L
T >= 0.128 nmol/L
p = 0.33
Percent survival
Progression-free survival (days)


D
Biopsy Site
Total Androgens
EDA2R
CIDEB
GSDMD
VSIG2
EFS
TRPS1
FNDC4
PIP4P2
PIGZ
TDRD10
SLC17A9
ZNF454
SLC2A1
BNIP3
ALDOC
STAP1
H2AC21
BMI1
LSM11
UNC13A
UNC79
SRRM3
RTKN2
MEST
Biopsy site
Bone
Lymph node
Other
Total Androgens

564

565

566 **Table 1 – Patient Characteristics, Disease History and Treatment History.**

567 Abbreviations: ADT – androgen deprivation therapy, IQR – interquartile range, PSA – prostate

568 specific antigen

	N	%	Median (IQR)	Range (min-max)
Age (years) at registration CIRCUS	29		67 (62 - 75)	48-81
WHO at registration CIRCUS	29		1 (0 - 1)	0-1
Plasma testosterone (nmol/L)	29		0.12 (0.05-0.2)	0.02 – 0.64
Prior ADT				
Drug-based	26	90		
Surgery-based (orchiectomy)	3	10		
With upfront docetaxel	5	17		
Prior systemic therapy (other than ADT)				
0	3	10		
1	5	17		
2	16	55		
3	4	14		
4	1	3		
Type of prior systemic therapy (other than ADT)				
Hormonal therapy[A] only	3	10		
Chemotherapy only	7	24		
Immunotherapy only	1	3		
Hormonal[A] and chemotherapy	12	41		
Chemotherapy and radionuclide therapy	1	3		
Hormonal[A], radionuclide and chemotherapy	1	3		
Hormonal[A], radionuclide and other therapy	1	3		
PSA at baseline (ug/L)[B]	34		75 (19.3 - 173.3)	1.5-913
PSA at progression (ug/L)[B]	34		123.5 (30.8 - 280.8)	5-1286

[A] Hormonal therapy included AR antagonists and abiraterone acetate with prednisone

[B] For some patients with multiple treatments progression and subsequent baseline sample was identical

569