Abiraterone Acetate for Cushing Syndrome: Study in a Canine Primary Adrenocortical Cell Culture Model

Karin Sanders,[1] Wesley L. de Wit,[1] Jan A. Mol,[1] Max Kurlbaum,[2,3] Sabine Kendl,[2,3] Matthias Kroiss,[2,3,4] Hans S. Kooistra,[1] and Sara Galac[1]

[1]Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, 3584 CM Utrecht, Netherlands; [2]University Hospital Würzburg, Core Unit Clinical Mass Spectrometry, 97080 Würzburg, Germany; [3]University Hospital Würzburg, Department of Internal Medicine I, Division of Endocrinology and Diabetology, 97080 Würzburg, Germany; and [4]Comprehensive Cancer Center Mainfranken, University of Würzburg, 97080 Würzburg, Germany

ORCiD numbers: 0000-0001-9634-9853 (K. Sanders); 0000-0002-4831-4995 (S. Galac).

Abiraterone acetate (AA) is a potent inhibitor of steroidogenic enzyme 17α-hydroxylase/17,20-lyase (CYP17A1). AA is approved for the treatment of prostate cancer but could also be used to treat patients with Cushing syndrome (CS). Similar to humans, canine glucocorticoid synthesis requires CYP17A1, providing a useful animal model. The objective of this study was to preclinically investigate the effect of AA on adrenocortical hormone production, cell viability, and mRNA expression of steroidogenic enzymes in canine primary adrenocortical cell cultures (n = 9) from the adrenal glands of nine healthy dogs. The cells were incubated with AA (0.125 nM to 10 μM) for 72 hours under basal conditions and with 100 nM ACTH(1-24). Adrenocortical hormone concentrations were measured in culture medium using liquid chromatography–mass spectrometry, RNA was isolated from cells for subsequent real-time quantitative PCR analysis, and cell viability was assessed with an alamarBlue™ assay. AA reduced cortisol (IC_{50}, 21.4 $\pm$ 4.6 nM) without affecting aldosterone under basal and ACTH-stimulated conditions. AA increased progesterone under basal and ACTH-stimulated conditions but reduced corticosterone under basal conditions, suggesting concurrent inhibition of 21-hydroxylation. AA did not affect the mRNA expression of steroidogenic enzymes and did not inhibit cell viability. In summary, primary canine adrenocortical cell culture is a useful model system for drug testing. For the treatment of CS, AA may to be superior to other steroidogenesis inhibitors due to its low toxicity. For future *in vivo* studies, dogs with endogenous CS may provide a useful animal model. (***Endocrinology*** **159: 3689–3698, 2018)**

Cushing syndrome (CS) is a serious endocrine disorder that results from chronic exposure to excessive glucocorticoids. Endogenous CS is most commonly (~70%) caused by an ACTH-producing pituitary adenoma (Cushing disease) and second most commonly (~20%) by a primary functional adrenocortical tumor (1, 2). When not properly treated, CS is associated with increased morbidity and mortality rates (3).

Medical treatment options target the pituitary (cabergoline, pasireotide), the adrenal glands (ketoconazole, metyrapone, mitotane), or the glucocorticoid receptor (mifepristone). Response to medical treatment targeting the adrenal gland is highly variable (4). All currently available medical treatments have serious shortcomings (*e.g.*, ketoconazole may lead to serious hepatitis, metyrapone may lead to disabling androgen and mineralocorticoid synthesis and requires frequent dosing, and mitotane has serious side effects and requires diligent monitoring) (5, 6). To expand the range of future treatment options, new medical agents with high selectivity and tolerability are needed.

ISSN Online 1945-7170

Received 15 June 2018. Accepted 7 September 2018.
First Published Online 13 September 2018

Abbreviations: AA, abiraterone acetate; CS, Cushing syndrome; CYP11A1, cytochrome P450 side chain cleavage; CYP11B1, 11β-hydroxylase; CYP17A1, 17α-hydroxylase/17,20-lyase; CYP21A2, 21-hydroxylase; D4A, Δ^4-abiraterone; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; DMSO, dimethyl sulfoxide; HSD3B2, 3β-hydroxysteroid dehydrogenase 2; LC-MS/MS, liquid chromatography–tandem mass spectrometry; mCRPC, metastatic castration-resistant prostate cancer; PR, progesterone receptor; RT-qPCR, quantitative RT-PCR.

An interesting target for selective inhibition of cortisol production is the steroidogenic enzyme 17α-hydroxylase/17,20-lyase (CYP17A1), which plays an important role in glucocorticoid synthesis but not in mineralocorticoid synthesis (7). This enzyme is also required for adrenal androgen production, which stimulates tumor growth in patients with metastatic castration-resistant prostate cancer (mCRPC) (8). To inhibit androgen synthesis in these patients, the potent CYP17A1 inhibitor abiraterone acetate (AA) was approved as treatment in 2011 in Europe and the United States (9, 10). Although generally well tolerated, AA induces hypocortisolism with a concomitant rise in circulating ACTH concentration in patients with mCRPC, which necessitates coadministration of glucocorticoids to reduce the associated side effects (11). Although the concurrent inhibition of cortisol production with AA is unintentional in patients with mCRPC, this offers interesting opportunities for the treatment of patients with CS.

Testing new treatment options for use in humans with CS is hampered by the limited presence of suitable cell culture systems and *in vivo* models. Most *in vitro* studies in the field have used the human adrenocortical carcinoma (ACC) NCI-H295 cell line. Only recently, three novel ACC cell lines have been described; however, these cell lines are available in only a few laboratories (12, 13). Clinical trials are impeded by the low estimated incidence of 0.5 to 5.0 cases per million people per year (14). In contrast, spontaneous CS is quite common in dogs, with an estimated incidence of 1 to 2.5 cases per 1000 dogs per year (15, 16). Canine CS shows remarkable similarities to human CS, including the etiology, clinical presentation, and treatment options, and can therefore be regarded as a spontaneous animal model for human CS (17, 18). Glucocorticoid synthesis in dogs also requires the AA target CYP17A1, which is not the case in, for example, adult rats, mice, and rabbits (19). To elucidate how AA affects adrenocortical cells, we hypothesized that an *in vitro* system with cultured canine primary adrenocortical cells may convey information that is useful for subsequent *in vivo* studies.

In this study, we evaluated whether and how AA affects the hormone production, mRNA expression of steroidogenic enzymes, and cell viability of canine primary adrenocortical cells under basal and ACTH-stimulated conditions.

Materials and Methods

Animals and tissues

The adrenal glands of 10 healthy dogs (one for a pilot experiment, nine for the following experiments) that were euthanized for reasons unrelated to the current study were used. The current study was approved by the Ethical Committee of Utrecht University. The median age was 17 months (range, 14 to 18 months), and the median body weight was 25 kg (range, 11 to 26 kg). Three dogs were male, and seven were female; all of the dogs were sexually intact. Seven dogs were of mixed breed; three dogs were beagles. The adrenal glands were collected within 10 minutes after euthanasia.

Primary cell cultures

Cell suspensions were prepared as described previously (20). In brief, the adrenal cortices were enzymatically digested in a collagenase solution, filtered, washed, and centrifuged. The cells were diluted to 1×10^5 cells/mL and seeded in Multiwell 96-well (100 μL per well) or 24-well (1 mL per well) plates (Primaria™; Corning, Amsterdam, Netherlands). The cells were cultured in a mixture of DMEM F-12 (Gibco, Invitrogen, Breda, Netherlands) with 1% Insulin-Transferrin-Selenium (Gibco), 0.125% BSA, 2.5% Nu-Serum (Corning), and 1% penicillin/streptomycin (Gibco). The cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2 and were left to attach for 4 to 6 days prior to compound incubations.

Cell treatments

AA (Sigma-Aldrich, Zwijndrecht, Netherlands) was dissolved in dimethyl sulfoxide (DMSO; Merck KGaA, Darmstadt, Germany). To determine the optimal incubation time, one adrenocortical cell culture was incubated for 24, 48, 72, and 96 hours in quadruplicate with increasing concentrations of AA (eight concentrations, 0.125 nM to 10 μM in fivefold dilution series) or with DMSO as vehicle control and coincubated with 100 nM synthetic ACTH(1-24) (Synacthen; Novartis Pharma BV, Arnhem, Netherlands) to induce hypercortisolism. The DMSO end concentration was 0.1% in all conditions. Cortisol concentrations were measured in the culture medium with RIA as described previously (21). To determine the IC_{50} and maximal inhibitory concentration of AA, nine adrenocortical cell cultures were incubated in quadruplicate with AA or with DMSO as vehicle control for 72 hours and coincubated with 100 nM synthetic ACTH(1-24). Cortisol concentrations were measured in the culture medium with RIA as described previously (21). In the following experiments, the cells were treated in duplicate [quantitative RT-PCR (RT-qPCR) and steroidomics] or quadruplicate (cell viability assay) with 16 nM (approximate IC_{50}) or 2 μM (maximal inhibition) of AA or with DMSO as vehicle control. The cells were coincubated with (ACTH-stimulated cell cultures) and without (basal conditions) 100 nM synthetic ACTH(1-24) to induce hypercortisolism.

Steroidomics

After 72 hours of incubation, steroid hormones were measured in the culture medium with liquid chromatography–tandem mass spectrometry (LC-MS/MS). The LC-MS/MS instrumentation consisted of a 6500+ QTRAP (SCIEX®, Framingham, MA) MS-system coupled to a 1290 HPLCsystem (G4226A autosampler, infinityBinPump, G1316C column-oven, G1330B thermostat; Agilent Technologies, Santa Clara, CA). Analysis was performed with a commercial kit (MassChrom-Steroids in Serum/Plasma®; Chromsystems®, Gräfelfing, Germany) for determination of 13 steroid hormones (MRM-Mode, isotope standards) according to the manufacturer's instruction. After an off-line solid-phase extraction extraction procedure of 500 μl culture medium, 5 μL were used for analysis. Concentrations were calculated with

Analyst® Software (1.6.3) via six-point calibration and 1/x weighting. Correctness of measurements was controlled by commercial quality controls and periodic participation in ring trails. Additional information on the measurement ranges, multiple reaction monitoring, recovery, and intraday and interday precision of the LC-MS/MS procedure is provided in an online repository (22). Cell-free culturing medium was also analyzed to determine whether hormones were present in the culture medium that did not originate from the cell cultures.

To relate the hormone concentrations to the number of cells per well, the protein concentration per well was measured using the Bradford Protein Assay (Bio-Rad, Veenendaal, Netherlands) according to the manufacturer's instructions. All hormone concentrations were calculated as nmol hormone per mg protein per well.

RT-qPCR

RNA was isolated from the cells after 48 hours of incubation by using the RNeasy Micro Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. After the RNA concentrations were measured with Nanodrop (ND-1000; Isogen Life Science, Utrecht, Netherlands), cDNA was synthesized with the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions and diluted to 1 ng/μL. mRNA expression was determined of steroidogenic acute regulatory protein, cytochrome P450 side chain cleavage (*CYP11A1*), *CYP17A1*, 3β-hydroxysteroid dehydrogenase 2 (*HSD3B2*), 21-hydroxylase (*CYP21A2*), and 11β-hydroxylase (*CYP11B1*) using SYBR-green supermix (Bio-Rad) in a CFX384 Touch Real-Time PCR Detection System (Bio-Rad) instrument. Primers and conditions for all genes were as described previously (19, 23). The results of two technical replicates were averaged. To correct for differences in cDNA concentrations, four reference genes were analyzed: tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta polypeptide, signal recognition particle receptor, ribosomal protein S19, and succinate dehydrogenase complex subunit A (24–26). GeNorm software (27) was used to analyze the reference genes' stability and variance within different cell cultures and different conditions, which justified their use. The $2^{-\Delta\Delta CT}$ method (28) was used to calculate the normalized relative mRNA expression of the target genes.

Cell viability

Cell viability was assessed after 72 hours of incubation by using the alamarBlue™ Cell Viability Reagent (Thermo Fisher Scientific, Breda, Netherlands) as described previously (20). In brief, the cells were incubated with the alamarBlue reagent in the dark at 37°C for 4 hours, after which fluorescence was measured at 535 nm excitation and 595 nm emission.

Statistical analysis

The IC_{50} values of AA on cortisol production measured with RIA were calculated with sigmoidal dose-response curves using SigmaPlot (Systat Software, San Jose, CA). All hormone concentrations that were nondetectable (specified in the text when applicable) were assigned a value of 0.5 times the quantification limit for statistical analysis. The data were tested for normality using the Shapiro-Wilk test. Normally distributed data (viability data) were analyzed with repeated measures ANOVA with a *post hoc* Bonferroni correction. Sphericity was checked with Mauchly test, which showed no significant variances of differences. Nongaussian data (RT-qPCR and hormone data) were analyzed with the Friedman test. Significant differences were analyzed *post hoc* with the Wilcoxon signed-rank test with a Bonferroni correction. P values <0.05 were considered significant. Data are reported as mean ± SEM of nine individual cell cultures unless indicated otherwise.

Results

Optimal incubation time and concentrations

In the pilot experiment AA most notably inhibited the cortisol production after 72 and 96 hours of incubation (Fig. 1A). To determine the IC_{50} and maximal inhibitory concentrations, nine adrenocortical cell cultures were incubated with increasing concentrations of AA and with ACTH for 72 hours (Fig. 1B). The mean IC_{50} of AA was 21.4 ± 4.6 nM (range, 3.0 to 39.8 nM). Because the mean IC_{50} most closely resembled 16 nM in the dilution series and the maximal inhibition of cortisol production was seen at 2000 nM AA, the following experiments were performed using 0, 16, and 2000 nM AA.

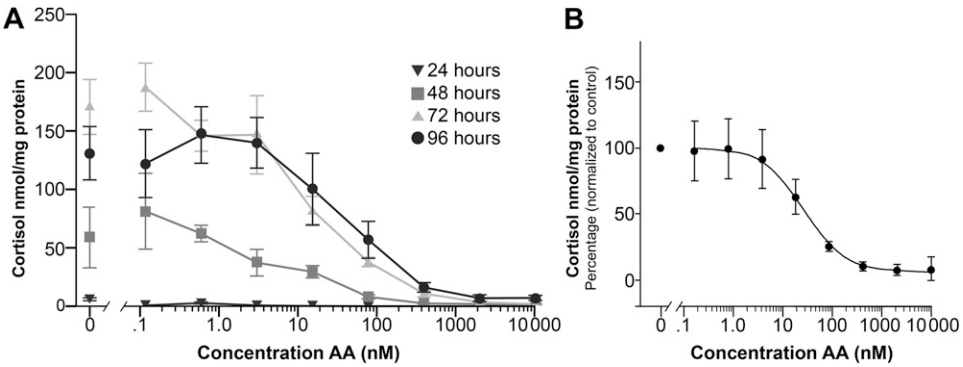

Figure 1. Determination of the optimal effects of AA on cortisol production as measured with radioimmunoassay. (A) To determine the optimal incubation time, canine primary adrenocortical cells (primary cell culture, n = 1) were incubated in quadruplicate with increasing concentrations of AA (0.125 nM to 10 μM) or with DMSO vehicle control and with 100 nM ACTH(1-24) for 24, 48, 72, and 96 hours. Data are shown as mean ± SEM cortisol/protein ratios. (B) To determine IC_{50} and maximal inhibitory concentration, canine primary adrenocortical cell cultures (primary cell cultures, n = 9) were incubated with increasing concentrations of AA (0.125 nM to 10 μM) or with DMSO vehicle control and with 100 nM ACTH(1-24) for 72 hours. Data are shown as mean ± SEM cortisol/protein ratios in percentage, normalized to the DMSO vehicle controls.

AA inhibits glucocorticoid production

AA at concentrations of 16 and 2000 nM significantly reduced the cortisol concentration in the culture medium of cell cultures under basal conditions (*i.e.*, without ACTH stimulation) and of ACTH-stimulated cell cultures (Fig. 2). Under basal conditions, the cortisol concentration was undetectable in three out of nine cell cultures incubated with 16 nM AA and in four out of nine cell cultures incubated with 2000 nM. The cortisol precursor 11-deoxycortisol concentration was dose-dependently reduced by AA under basal conditions and by 2000 nM in the ACTH-stimulated cell cultures (Fig. 2). The concentration of cortisone was reduced by both AA concentrations under basal conditions and dose-dependently in the ACTH-stimulated cell cultures (Fig. 2).

AA inhibits corticosterone production only under basal conditions but does not affect aldosterone production

Although CYP17A1 is not required for corticosterone production, 2000 nM AA significantly reduced the corticosterone concentration in cell cultures under basal conditions (Fig. 2). In contrast, in the ACTH-stimulated cell cultures, AA slightly but significantly increased the corticosterone concentration at 16 nM but did not affect the corticosterone concentration at 2000 nM (Fig. 2).

The aldosterone concentration was unaffected by AA under both basal and ACTH-stimulated conditions (Fig. 2).

AA increases progesterone production and inhibits 17-OH–progesterone production

The concentration of progesterone, a substrate of CYP17A1, was dose-dependently increased by AA under both basal and ACTH-stimulated conditions (Fig. 2). Subsequent 17-hydroxylation of progesterone to 17-OH–progesterone was inhibited by AA because AA dose-dependently reduced the 17-OH–progesterone concentration in the cell cultures under basal conditions and at 2000 nM in the ACTH-stimulated cell cultures (Fig. 2).

AA inhibits androstenedione production when stimulated with ACTH

The concentration of androstenedione, an adrenal androgen precursor, was significantly reduced in cell cultures under basal conditions by 16 nM AA but was unaffected by 2000 nM AA (Fig. 2). In the ACTH-stimulated cell cultures, AA dose-dependently reduced the androstenedione concentration (Fig. 2). The androstenedione concentration was undetectable in four out of nine ACTH-stimulated cell cultures incubated with 2000 nM AA.

The concentrations of dehydroepiandrosterone (DHEA), DHEA sulfate (DHEAS), estradiol, testosterone, and DHT were measured, but their concentrations were either below the detection limit (DHEA, DHEAS, DHT) or similar to the concentration that was measured in cell-free culture medium (estradiol, testosterone) in all or in the majority of the cell cultures and were therefore excluded from further analyses.

AA inhibits 17-hydroxylation, 21-hydroxylation, and 11β-hydroxylation

To determine why AA increased the concentration of progesterone but not of corticosterone, we performed additional measurements of 11-deoxycorticosterone in a small number of key samples (single measurements of 0, 16, and 2000 nM conditions of four adrenocortical cell cultures under basal and ACTH-stimulated conditions). Under basal conditions, AA reduced the mean 11-deoxycorticosterone concentration (0 nM AA, 12.9 $\pm$ 5.5 nmol/mg protein; 2000 nM AA, 4.8 $\pm$ 0.9 nmol/mg protein), whereas under ACTH-stimulated conditions AA increased the mean 11-deoxycorticosterone concentration (0 nM AA, 16.8 $\pm$ 10.9 nmol/mg protein; 2000 nM AA, 33.5 $\pm$ 3.1 nmol/mg protein). We calculated the ratios relative to the 0 nM control of 17-OH–progesterone/progesterone (17-hydroxylase), 11-deoxycorticosterone/progesterone (21-hydroxylase), and corticosterone/11-deoxycorticosterone (11β-hydroxylase) (Fig. 3). As expected, 2000 nM AA substantially decreased the 17-OH–progesterone/progesterone ratio (17-hydroxylase) for adrenocortical cell cultures under basal and ACTH-stimulated conditions. In addition, to a lesser extent AA decreased the ratios of 11-deoxycorticosterone/progesterone (21-hydroxylase) and corticosterone/11-deoxycorticosterone (11β-hydroxylase).

The mRNA expression of steroidogenic enzymes

The AA target *CYP17A1* was expressed in all cell cultures under basal conditions, and the addition of ACTH significantly increased its expression (Table 1). The same was true for the other key steroidogenic enzymes.

mRNA expression of the steroidogenic enzymes was unaffected by either AA concentration, except for that of *CYP11A1* under basal cell culture conditions, which was significantly higher at 2000 nM AA (Fig. 4).

AA does not inhibit cell viability

The cell viability was not inhibited by AA under basal (Fig. 5A) or ACTH-stimulated (Fig. 5B) conditions.

Discussion

To expand the range of treatment options for patients with CS, new drugs that inhibit glucocorticoid production with high selectivity and tolerability are required. This study contributes to the current knowledge on the mechanism of action of AA, and our findings warrant future studies to

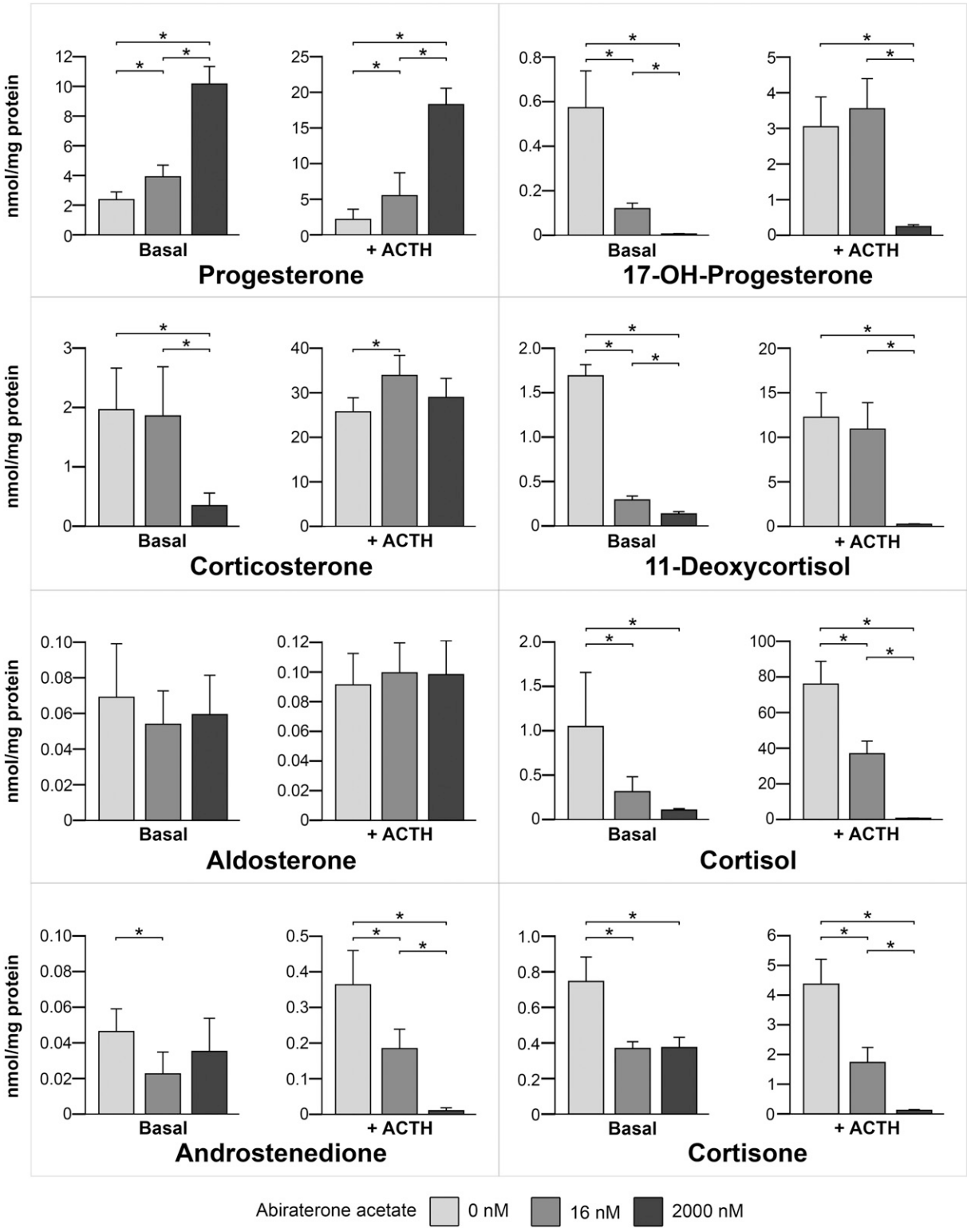

Figure 2. The effects of AA on steroid hormone production as measured with LC-MS/MS. Steroid hormone production was measured in canine primary adrenocortical cell cultures (n = 9) under basal and ACTH-stimulated conditions. Data are shown as mean ± SEM of nanomole of hormone per milligram protein. Scale bars are adjusted to each hormone concentration to aid visualization. $*P < 0.05$.

determine whether AA is a potential treatment option for both human patients and canines with endogenous CS.

In a previous study, we showed that cultured canine primary adrenocortical cells retain their ability to produce cortisol and to respond to ACTH (20) and are therefore a useful *in vitro* model to study adrenocortical hormone secretion. In contrast, the commonly used cell line NCI-H295R responds only mildly to ACTH stimulation (29), and these cells are therefore an imperfect model to study adrenocortical hormone secretion or, in

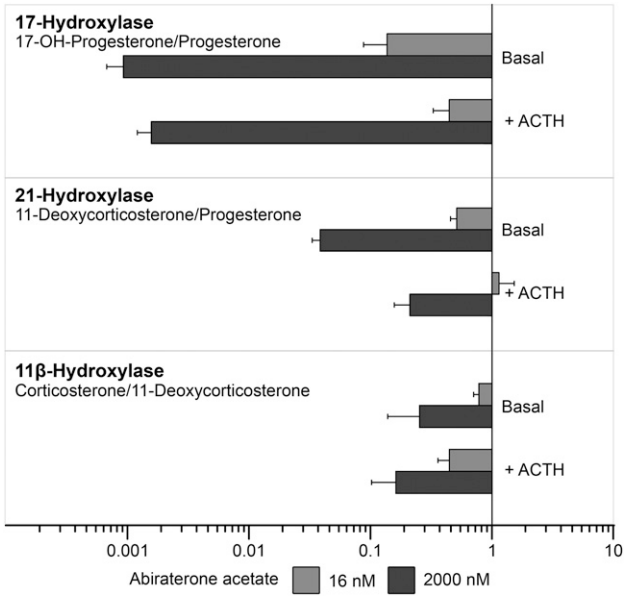

Figure 3. The effects of AA on steroid hormone ratios. Steroid hormone production was measured with LC-MS/MS in canine primary adrenocortical cell cultures (n = 4) under basal and ACTH-stimulated conditions. Ratios were calculated between the indicated hormones. The results were calculated in fold change relative to the 0 nM AA control. Data are shown as mean ± SEM fold change.

this case, to determine how ACTH stimulation affects the mechanism of action of AA. Moreover, a single cell line is not necessarily a good representation of the more heterogeneous adrenal cortex *in vivo*. The difference in the IC_{50} values of AA that we observed between individual cell cultures (lowest IC_{50}, 3.0 nM; highest, 39.8 nM) confirms the heterogeneous nature of primary cell culture, which could therefore be a better predictor of *in vivo* functionality. This variation in IC_{50} values could be related to multiple factors (*e.g.*, intracellular substrate availability, esterase activity, enzyme expression, and enzyme cofactor presence) because the combination of these factors can be different in each cell culture.

Table 1. mRNA Expression of Steroidogenic Enzymes in Canine Primary Adrenocortical Cell Cultures (n = 9) Under Basal Conditions in ΔCT and Fold Change After Stimulation With 100 nM ACTH(1-24)

Gene	ΔCT Basal	Fold Change + ACTH	*P* Value + ACTH
StAR	1.44 ± 0.37	15.4 ± 3.5	0.012
CYP11A1	2.88 ± 0.33	10.9 ± 2.5	0.012
CYP17A1	6.87 ± 0.44	106.7 ± 29.3	0.008
HSD3B2	1.37 ± 0.47	23.0 ± 7.1	0.008
CYP21A2	5.70 ± 0.43	33.9 ± 9.2	0.012
CYP11B1	10.62 ± 0.58	27.0 ± 8.3	0.018

Data are shown as mean ± SEM relative to the geometric mean CT value of the reference genes (ΔCT basal) or as fold change relative to the expression under basal conditions (fold change + ACTH).

Abbreviations: CT, cycle threshold; StAR, steroidogenic acute regulatory protein.

An advantage of using canine over, for example, murine adrenocortical cells is that they require and express CYP17A1 for glucocorticoid production and therefore produce the same glucocorticoids as humans (19), which is important when testing CYP17A1 inhibitors such as AA. The cultured cells in the current study indeed expressed mRNA of the AA target *CYP17A1*, which was greatly upregulated by ACTH. The inhibition of this enzyme by AA substantially reduced the cortisol and other glucocorticoid concentrations in this study, which is an important goal of treatment in CS.

Although CYP17A1 is not required for corticosterone production, AA also significantly reduced the corticosterone concentration in the cell cultures under basal conditions, which implies that AA also inhibits 21-hydroxylation or 11β-hydroxylation. Indeed, recent studies have shown that AA inhibits the 21-hydroxylase activity of CYP21A2 in NCI-H295R cells (30, 31). To determine whether this inhibition of 21-hydroxylase occurred in the canine adrenocortical cells, we performed additional measurements in a small number of samples that included 11-deoxycorticosterone measurements. Although the limited number of samples hampers statistical analyses, under basal conditions AA increased progesterone but decreased 11-deoxycorticosterone, suggesting that 21-hydroxylation is indeed inhibited. The calculation of the 11-deoxycorticosterone/progesterone ratios suggests that, in cell cultures under both basal and ACTH-stimulated conditions, 21-hydroxylase and 11β-hydroxylase were inhibited by AA. AA has also been shown to inhibit the human HSD3B2 enzyme (32). In this study, we were not able to measure all required hormones to test whether this was also the case in the canine adrenocortical cells. However, the substantially and significantly increased progesterone concentration, which is a product of HSD3B2 conversion, suggest that if HSD3B2 is indeed inhibited, its capacity remains extensive.

AA is the ester prodrug of abiraterone. AA gets rapidly deacetylated to the active form abiraterone *in vivo*, and its bioavailability strongly depends on whether it is taken with food (33, 34). Abiraterone can be converted to the active metabolite Δ^4-abiraterone (D4A) by HSD3B isoforms (35). In the culture medium of two of the adrenocortical cell cultures described in this study, the percentage of the D4A metabolite that we could detect was 13% to 38% of the added 2000 nM AA [preliminary data, method similar to that described by van Nuland *et al.* (36)]. AA supposedly is deacetylated to abiraterone in our cell culture system by nonspecific esterases. Because the canine adrenocortical cells highly express *HSD3B2* (Table 1), this enzyme presumably converts a

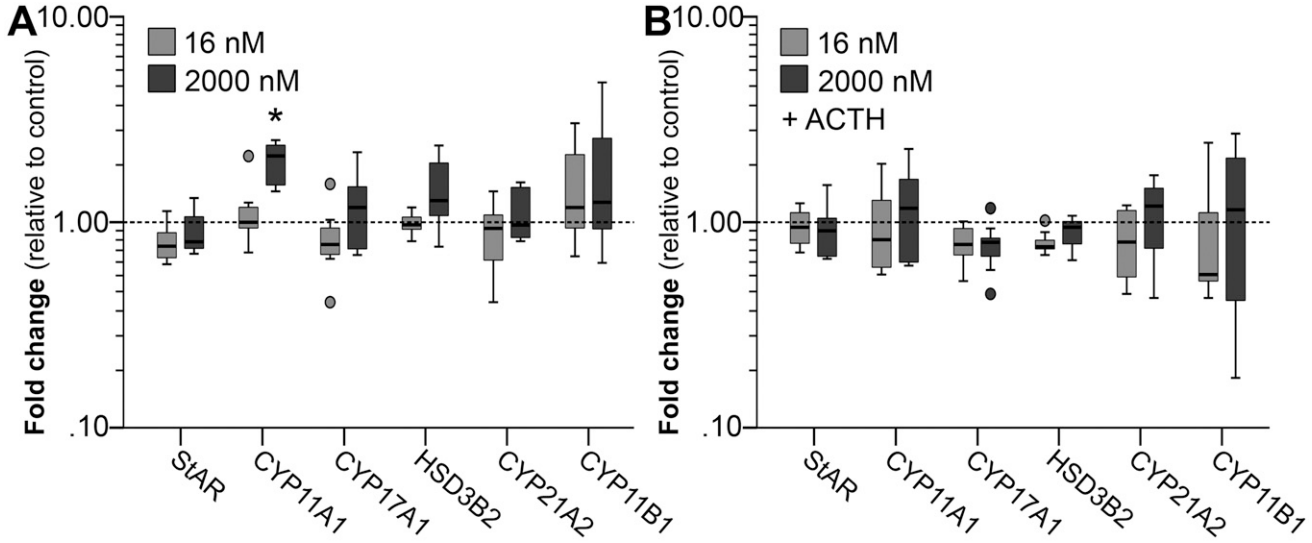

Figure 4. The effects of AA on the mRNA expression of steroidogenic enzymes. Gene expression was measured in canine primary adrenocortical cell cultures (n = 9) under (A) basal and (B) ACTH-stimulated conditions. Data are shown as mean ± SEM fold change relative to the corresponding DMSO vehicle control. The dotted line represents control. The circles above and below the box plots indicate outliers. $*P < 0.05$. StAR, steroidogenic acute regulatory protein.

large portion of the released abiraterone to D4A. This D4A metabolite has been shown to inhibit CYP21A2 (37), which might contribute to the inhibition of 21-hydroxylase.

When AA was administered without concurrent glucocorticoids to patients with advanced castration-resistant prostate cancer, the decreased glucocorticoid production resulted in substantially increased plasma ACTH concentrations (11). This in turn markedly increased steroids upstream of CYP17A1 that can exert mineralocorticoid activity (*e.g.*, 11-deoxycorticosteron), which can lead to the mineralocorticoid excess syndrome. Because of subsequent suppression of the renin-angiotensin-aldosterone system, aldosterone itself did not increase. Coadministration of glucocorticoids inhibited the increased ACTH secretion and was thereby able to overcome the adverse effects caused by mineralocorticoid excess syndrome in most patients (11). Although 2000 nM AA decreased the mean 11-deoxycorticosterone concentration in our culture system under basal conditions, it increased the mean 11-deoxycorticosterone concentration when stimulated with ACTH (n = 4). This effect might be greater *in vivo* when the ACTH secretion increases due to a low cortisol concentration. However, whereas the goal of treatment in patients with castration-resistant prostate cancer is to completely block androgen production, the goal of treatment in patients with CS would be to achieve normocortisolism. Nevertheless, the potential increase in ACTH secretion *in vivo* warrants caution and possibly requires coadministration of other drugs, such as aldosterone receptor antagonists (*e.g.*, spironolactone, eplerenone) or epithelial sodium channel inhibitors (*e.g.*, amiloride) (38, 39).

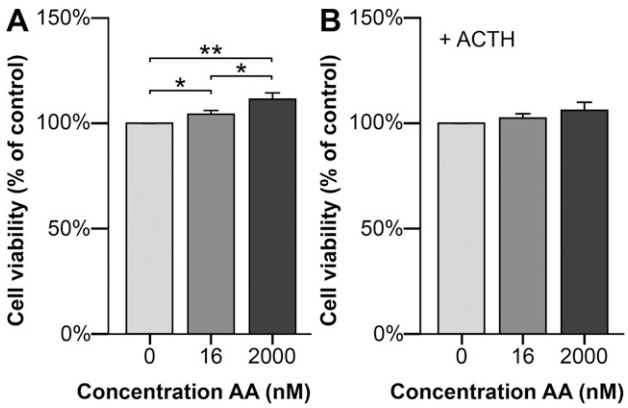

Figure 5. The effects of AA on cell viability. Cell viability was measured with alamarBlue™ assay in canine primary adrenocortical cell cultures (n = 9) under (A) basal and (B) ACTH-stimulated conditions. Data are shown as mean ± SEM of the percentage of detected fluorescence with each DMSO vehicle control (0 nM AA) set to 100%. $*P < 0.05$; $**P < 0.01$.

Recently, Fiorentini *et al.* (40) reported that AA inhibited the cell viability of the human ACC cell line NCI-H295R and of primary cell cultures derived from ACCs that were hormonally active but not from those that were hormonally inactive or of the hormonally inactive SW13 cell line. When they cultured the cells in the presence of the progesterone receptor (PR) antagonist mifepristone or when they silenced the *PR* gene, the inhibition of cell viability was prevented, indicating that the increased progesterone concentration has a role in the inhibition of cell viability through the PR (40). In contrast, in our current study, cell viability was not inhibited but slightly increased in the cell cultures under basal conditions, possible due to a compensatory increased cellular mitochondrial activity. The lack of inhibition of

cell viability was not caused by a lack of hormone production because these cells were hormonally active with also dramatically increased progesterone concentrations after AA incubation. Earlier work by our group showed that the canine adrenal cortex does express the *PR* (41). There are two PR isoforms that are synthesized from the *PR* gene in both humans and dogs: PRA and PRB. In NCI-H295R cells, the predominant isoform of the PR is PRB (40), whereas in dogs PRB has been shown to have very limited transactivation potential (42). This difference in isoform activity between the two species could explain the contradictory results. An alternative explanation for the contradictory effects of AA on cell viability is that healthy adrenocortical cells possibly respond differently to a high progesterone concentration than ACC cells.

In the cell cultures under basal conditions, 2000 nM AA significantly increased the mRNA expression of *CYP11A1*. Because this condition contained the lowest cortisol concentration in the culture medium, we hypothesize that this increase in *CYP11A1* expression might be related to an intra-adrenal feedback regulation of cortisol production. Although relatively little is known about the intra-adrenal feedback mechanism, a recent study provided evidence that cortisol can inhibit its own production (43). In the cell cultures under basal conditions, the cortisol concentrations inhibited by AA were in every case lower than in the ACTH-stimulated cell cultures where the *CYP11A1* expression was not increased by AA. This suggests that the increase in *CYP11A1* expression was not due to AA itself but was due to the low cortisol concentration. Considering that the other steroidogenic enzymes were not or not yet increased by 2000 nM AA in the adrenocortical cell cultures under basal conditions, *CYP11A1* seems most sensitive to the negative feedback of cortisol (or lack thereof), possibly because CYP11A1 is a rate-limiting enzyme in steroidogenesis (44).

Although 16 nM AA reduced the androstenedione concentration in the cell cultures under basal conditions, no significant reduction was seen with 2000 nM AA. A possible explanation for this is that AA inhibits DHEA sulfonation by the sulfotransferase 2A1 enzyme (45), which could increase the relative amount of DHEA that will be directly converted to androstenedione. The DHEA and DHEAS concentrations could not be detected in the current study, so we were not able to test this hypothesis. In the ACTH-stimulated cell cultures, androstenedione was inhibited substantially by AA, as also reported *in vivo* in women with 21-hydroxylase deficiency treated with AA (46). An advantage of concurrent inhibition of androgen production is that an ACC often co-secretes glucocorticoids and androgens (47), in which case administration of AA could inhibit the virilization symptoms (48).

Although many similarities exist between humans and dogs in terms of CS and adrenal physiology, there are some differences. For example, whereas humans have two different isoforms of the cytochrome P450 11B enzyme to catalyze the last steps in cortisol (CYP11B1) and aldosterone (aldosterone synthase; CYP11B2) synthesis, dogs appear to have only one functional isoform of this enzyme (19). It is therefore prudent to keep these species-related differences in mind when interpreting the results of this study.

In summary, the results of this study show that AA effectively inhibits cortisol production but does not affect aldosterone production. Future studies are warranted to determine whether AA can be a valuable addition to the current treatment options for both human and canine CS. To determine the *in vivo* efficacy, tolerability, and optimal dosing of AA, dogs with spontaneous CS may provide a useful animal model.

Acknowledgments

The authors thank Merel van Nuland and Jos H. Beijnen (Antoni van Leeuwenhoekziekenhuis, Amsterdam, Netherlands) for the D4A measurements and Adri Slob (Utrecht University, Utrecht, Netherlands) for RIA cortisol measurements and technical assistance.

Financial Support: This study was supported by the Deutsche Forschungsgemeinschaft (DFG) within the CRC/Transregio 205/1 "The Adrenal: Central Relay in Health and Disease" (Project B16) to M. Kroiss and an individual research grant from the DFG to M. Kroiss (KR 4371/1-2).

Correspondence: Sara Galac, DVM, PhD, Yalelaan 108, 3584 CM Utrecht, Netherlands. E-mail: s.galac@uu.nl.

Disclosure Summary: The authors have nothing to disclose.

References

1. Creemers SG, Hofland LJ, Lamberts SW, Feelders RA. Cushing's syndrome: an update on current pharmacotherapy and future directions. *Expert Opin Pharmacother*. 2015;**16**(12):1829–1844.
2. Cuevas-Ramos D, Lim DST, Fleseriu M. Update on medical treatment for Cushing's disease. *Clin Diabetes Endocrinol*. 2016;**2**(1):16.
3. Dekkers OM, Horváth-Puhó E, Jørgensen JOL, Cannegieter SC, Ehrenstein V, Vandenbroucke JP, Pereira AM, Sørensen HT. Multisystem morbidity and mortality in Cushing's syndrome: a cohort study. *J Clin Endocrinol Metab*. 2013;**98**(6):2277–2284.
4. Gadelha MR, Vieira Neto L. Efficacy of medical treatment in Cushing's disease: a systematic review. *Clin Endocrinol (Oxf)*. 2014;**80**(1):1–12.
5. Igaz P, Tömböl Z, Szabó PM, Likó I, Rácz K. Steroid biosynthesis inhibitors in the therapy of hypercortisolism: theory and practice. *Curr Med Chem*. 2008;**15**(26):2734–2747.

6. Fleseriu M, Castinetti F. Updates on the role of adrenal steroidogenesis inhibitors in Cushing's syndrome: a focus on novel therapies. *Pituitary*. 2016;**19**(6):643–653.
7. Auchus RJ. Steroid 17-hydroxylase and 17,20-lyase deficiencies, genetic and pharmacologic. *J Steroid Biochem Mol Biol*. 2017; **165**(Pt A):71–78.
8. Gomez L, Kovac JR, Lamb DJ. CYP17A1 inhibitors in castration-resistant prostate cancer. *Steroids*. 2015;**95**:80–87.
9. Gravanis I, Lopez AS, Hemmings RJ, Jiménez JC, Garcia-Carbonero R, Gallego IG, Giménez EV, O'Connor D, Giuliani R, Salmonson T, Pignatti F. The European medicines agency review of abiraterone for the treatment of metastatic castration-resistant prostate cancer in adult men after docetaxel chemotherapy and in chemotherapy-naive disease: summary of the scientific assessment of the committee for medicinal products for human use. *Oncologist*. 2013;**18**(9):1032–1042.
10. Garcia JA, Rini BI. Castration-resistant prostate cancer: many treatments, many options, many challenges ahead. *Cancer*. 2012; **118**(10):2583–2593.
11. Attard G, Reid AHM, Auchus RJ, Hughes BA, Cassidy AM, Thompson E, Oommen NB, Folkerd E, Dowsett M, Arlt W, de Bono JS. Clinical and biochemical consequences of CYP17A1 inhibition with abiraterone given with and without exogenous glucocorticoids in castrate men with advanced prostate cancer. *J Clin Endocrinol Metab*. 2012;**97**(2):507–516.
12. Hantel C, Shapiro I, Poli G, Chiapponi C, Bidlingmaier M, Reincke M, Luconi M, Jung S, Beuschlein F. Targeting heterogeneity of adrenocortical carcinoma: Evaluation and extension of preclinical tumor models to improve clinical translation. *Oncotarget*. 2016; **7**(48):79292–79304.
13. Kiseljak-Vassiliades K, Zhang Y, Bagby SM, Kar A, Pozdeyev N, Xu M, Gowan K, Sharma V, Raeburn CD, Albuja-Cruz M, Jones KL, Fishbein L, Schweppe RE, Somerset H, Pitts TM, Leong S, Wierman ME. Development of new preclinical models to advance adrenocortical carcinoma research. *Endocr Relat Cancer*. 2018; **25**(4):437–451.
14. Lacroix A, Feelders RA, Stratakis CA, Nieman LK. Cushing's syndrome. *Lancet*. 2015;**386**(9996):913–927.
15. Willeberg P, Priester W. Epidemiological aspects of clinical hyperadrenocorticism in dogs (canine Cushing's syndrome). *J Am Anim Hosp Assoc*. 1982;**18**(Sept/Oct):717–723.
16. O'Neill DG, Scudder C, Faire JM, Church DB, McGreevy PD, Thomson PC, Brodbelt DC. Epidemiology of hyperadrenocorticism among 210,824 dogs attending primary-care veterinary practices in the UK from 2009 to 2014. *J Small Anim Pract*. 2016;**57**(7):365–373.
17. Galac S, Reusch CE, Kooistra HS, Rijnberk A. Adrenals. In: Rijnberk A, Kooistra HS, eds. Clinical Endocrinology of Dogs and Cats. 2nd ed. Hannover, Germany: Schlütersche; 2010:93–154.
18. Galac S, Kool MMJ, Naan EC, Daminet S, Mol JA, Kooistra HS. Expression of the ACTH receptor, steroidogenic acute regulatory protein, and steroidogenic enzymes in canine cortisol-secreting adrenocortical tumors. *Domest Anim Endocrinol*. 2010;**39**(4): 259–267.
19. Sanders K, Mol JA, Kooistra HS, Slob A, Galac S. New insights in the functional zonation of the canine adrenal cortex. *J Vet Intern Med*. 2016;**30**(3):741–750.
20. Sanders K, Mol JA, Slob A, Kooistra HS, Galac S. Steroidogenic factor-1 inverse agonists as a treatment option for canine hypercortisolism: in vitro study. *Domest Anim Endocrinol*. 2018;**63**: 23–30.
21. Meijer JC, de Bruijne JJ, Rijnberk A, Croughs RJM. Biochemical characterization of pituitary-dependent hyperadrenocorticism in the dog. *J Endocrinol*. 1978;**77**(1):111–118.
22. Kurlbaum M, Kendl S, Kroiss M. Data from: Supplemental data steroidomics LC-MS/MS. Open Science Framework 2018. Deposited 3 September 2018. https://doi.org/10.17605/OSF.IO/SQJWK.
23. Bhatti SFM, Rao NAS, Okkens AC, Mol JA, Duchateau L, Ducatelle R, van den Ingh TSGAM, Tshamala M, Van Ham LML, Coryn M, Rijnberk A, Kooistra HS. Role of progestin-induced mammary-derived growth hormone in the pathogenesis of cystic endometrial hyperplasia in the bitch. *Domest Anim Endocrinol*. 2007;**33**(3):294–312.
24. Stassen QEM, Riemers FM, Reijmerink H, Leegwater PAJ, Penning LC. Reference genes for reverse transcription quantitative PCR in canine brain tissue. *BMC Res Notes*. 2015;**8**(1):761.
25. Schlotter YM, Veenhof EZ, Brinkhof B, Rutten VPMG, Spee B, Willemse T, Penning LC. A GeNorm algorithm-based selection of reference genes for quantitative real-time PCR in skin biopsies of healthy dogs and dogs with atopic dermatitis. *Vet Immunol Immunopathol*. 2009;**129**(1-2):115–118.
26. van Rijn SJ, Riemers FM, van den Heuvel D, Wolfswinkel J, Hofland L, Meij BP, Penning LC. Expression stability of reference genes for quantitative RT-PCR of healthy and diseased pituitary tissue samples varies between humans, mice, and dogs. *Mol Neurobiol*. 2014;**49**(2):893–899.
27. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol*. 2002;**3**(7):H0034.
28. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Δ Δ C(T)) method. *Methods*. 2001;**25**(4):402–408.
29. Wang T, Rainey WE. Human adrenocortical carcinoma cell lines. *Mol Cell Endocrinol*. 2012;**351**(1):58–65.
30. Udhane SS, Dick B, Hu Q, Hartmann RW, Pandey AV. Specificity of anti-prostate cancer CYP17A1 inhibitors on androgen biosynthesis. *Biochem Biophys Res Commun*. 2016;**477**(4): 1005–1010.
31. Malikova J, Brixius-Anderko S, Udhane SS, Parween S, Dick B, Bernhardt R, Pandey AV. CYP17A1 inhibitor abiraterone, an anti-prostate cancer drug, also inhibits the 21-hydroxylase activity of CYP21A2. *J Steroid Biochem Mol Biol*. 2017;**174**:192–200.
32. Li R, Evaul K, Sharma KK, Chang KH, Yoshimoto J, Liu J, Auchus RJ, Sharifi N. Abiraterone inhibits 3β-hydroxysteroid dehydrogenase: a rationale for increasing drug exposure in castration-resistant prostate cancer. *Clin Cancer Res*. 2012; **18**(13):3571–3579.
33. Barrie SE, Potter GA, Goddard PM, Haynes BP, Dowsett M, Jarman M. Pharmacology of novel steroidal inhibitors of cytochrome P450(17) alpha (17 alpha-hydroxylase/C17-20 lyase). *J Steroid Biochem Mol Biol*. 1994;**50**(5-6):267–273.
34. Stappaerts J, Geboers S, Snoeys J, Brouwers J, Tack J, Annaert P, Augustijns P. Rapid conversion of the ester prodrug abiraterone acetate results in intestinal supersaturation and enhanced absorption of abiraterone: in vitro, rat in situ and human in vivo studies. *Eur J Pharm Biopharm*. 2015;**90**:1–7.
35. Li Z, Bishop AC, Alyamani M, Garcia JA, Dreicer R, Bunch D, Liu J, Upadhyay SK, Auchus RJ, Sharifi N. Conversion of abiraterone to D4A drives anti-tumour activity in prostate cancer. *Nature*. 2015;**523**(7560):347–351.
36. van Nuland M, Rosing H, de Vries J, Ovaa H, Schellens JHM, Beijnen JH. An LC-MS/MS method for quantification of the active abiraterone metabolite Δ(4)-abiraterone (D4A) in human plasma. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2017;**1068-1069**: 119–124.
37. Yoshimoto FK, Peng HM, Zhang H, Anderson SM, Auchus RJ. Epoxidation activities of human cytochromes P450c17 and P450c21. *Biochemistry*. 2014;**53**(48):7531–7540.
38. Bedussi F, Galli D, Fragni M, Valcamonico F, Rossini E, Dalla Volta A, Vezzoli S, Roca E, Ferrari V, Lazzari B, Memo M, Sigala S, Berruti A. Amiloride is effective in the management of abiraterone-induced mineralocorticoid excess syndrome without interfering with its antineoplastic activity. *Pharmacology*. 2017;**100**(5-6): 261–268.

39. Gill D, Gaston D, Bailey E, Hahn A, Gupta S, Batten J, Alex A, Boucher K, Stenehjem D, Agarwal N. Efficacy of eplerenone in the management of mineralocorticoid excess in men with metastatic castration-resistant prostate cancer treated with abiraterone without prednisone. *Clin Genitourin Cancer*. 2017;**15**(4):e599–e602.
40. Fiorentini C, Fragni M, Perego P, Vezzoli S, Bonini SA, Tortoreto M, Galli D, Claps M, Tiberio GA, Terzolo M, Missale C, Memo M, Procopio G, Zaffaroni N, Berruti A, Sigala S. Antisecretive and antitumor activity of abiraterone acetate in human adrenocortical cancer: a preclinical study. *J Clin Endocrinol Metab*. 2016;**101**(12): 4594–4602.
41. Kool MMJ, Galac S, van der Helm N, Spandauw CG, Kooistra HS, Mol JA. Expression of somatostatin, dopamine, progesterone and growth hormone receptor mRNA in canine cortisol-secreting adrenocortical tumours. *Vet J*. 2015;**206**(1):108–110.
42. Gracanin A, Voorwald FA, Van Wolferen M, Timmermans-Sprang E, Mol JA. Marginal activity of progesterone receptor B (PR-B) in dogs but high incidence of mammary cancer. *J Steroid Biochem Mol Biol*. 2014;**144**(Part B):492–499.
43. Walker JJ, Spiga F, Gupta R, Zhao Z, Lightman SL, Terry JR. Rapid intra-adrenal feedback regulation of glucocorticoid synthesis. *J R Soc Interface*. 2015;**12**(102):20140875.
44. Miller WL, Auchus RJ. The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. *Endocr Rev*. 2011;**32**(1):81–151.
45. Yip CKY, Bansal S, Wong SY, Lau AJ. Identification of galeterone and abiraterone as inhibitors of dehydroepiandrosterone sulfonation catalyzed by human hepatic cytosol, SULT2A1, SULT2B1b, and SULT1E1. *Drug Metab Dispos*. 2018;**46**(4):470–482.
46. Auchus RJ, Buschur EO, Chang AY, Hammer GD, Ramm C, Madrigal D, Wang G, Gonzalez M, Xu XS, Smit JW, Jiao J, Yu MK. Abiraterone acetate to lower androgens in women with classic 21-hydroxylase deficiency. *J Clin Endocrinol Metab*. 2014;**99**(8): 2763–2770.
47. Alesini D, Iacovelli R, Palazzo A, Altavilla A, Risi E, Urbano F, Manai C, Passaro A, Magri V, Cortesi E. Multimodality treatment of gynecomastia in patients receiving antiandrogen therapy for prostate cancer in the era of abiraterone acetate and new antiandrogen molecules. *Oncology*. 2013;**84**(2):92–99.
48. Claps M, Lazzari B, Grisanti S, Ferrari V, Terzolo M, Sigala S, Vezzoli S, Memo M, Castellano M, Berruti A. Management of severe Cushing syndrome induced by adrenocortical carcinoma with abiraterone acetate: a case report. *AACE Clin Case Rep*. 2016;**2**(4):337–341.