CANINE CUSHING'S SYNDROME:

Prognostic Factors and New Treatment Options

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Canine Cushing's Syndrome:
Prognostic Factors and New Treatment Options

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Nieuwe Behandelingsmogelijkheden
(met een samenvatting in het Nederlands)

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Chapter

1

Aims and Scope

Hypercortisolism, often referred to as Cushing's syndrome, is one of the most common endocrine disorders in dogs. It is caused by an ACTH-secreting pituitary tumor (pituitary-dependent hypercortisolism; PDH) in ~80-85% of cases, and by a cortisol-secreting adrenocortical tumor (ACT) in ~15-20% of cases. The cortisol excess itself can be detrimental, but so can the mass-occupying effects of a pituitary tumor or an ACT, or the metastases of an ACT. Therefore, surgical removal of the causal tumor is a good treatment option. Alternatively, pharmacotherapy can be used to eliminate the clinical signs of hypercortisolism. In dogs with PDH the steroidogenesis inhibitor trilostane is often used, and in dogs with an ACT either trilostane or the adrenocorticolytic drug mitotane can be used. However, both treatment options have disadvantages. The current treatment options for canine Cushing syndrome are reviewed in **Chapter 2**. To expand the range of treatment options, new medical agents with high selectivity and tolerability are desired.

To know which steroidogenic enzymes can be targeted for selective inhibition of cortisol production, first we must know which steroidogenic enzymes are present in the canine adrenal cortex and which are specific for cortisol production. Because dogs produce the same mineralocorticoid and glucocorticoid hormones as humans do, it was assumed that they would also express the same set of steroidogenic enzymes. However, it was never studied whether this was really the case. The aim of the study in **Chapter 3** was therefore to investigate the zonal expression of steroidogenic enzymes in the canine adrenal cortex.

Based on the results in Chapter 3, we concluded that an interesting target to selectively inhibit cortisol production would be the steroidogenic enzyme 17α-hydroxylase/17,20-lyase (CYP17A1). Consequently, we looked for potential CYP17A1 inhibitors, and the candidate we found was abiraterone acetate (AA). AA is a CYP17A1 inhibitor that has been approved to inhibit androgen synthesis in humans with metastatic castration-resistant prostate cancer, in whom it also induces hypocortisolism. In **Chapter 4** we investigated the effects of AA on adrenocortical hormone production, mRNA expression of steroidogenic enzymes, and cell viability of canine primary adrenocortical cells.

Another potential treatment target to inhibit cortisol production in dogs with PDH would be the receptor for ACTH: the $G_{s\alpha}$-protein coupled melanocortin 2 receptor (MC2R). Binding of ACTH to the MC2R results in activation of the cAMP-protein kinase A

signaling pathway, which eventually results in increased cortisol production. A potent MC2R antagonist would therefore be an interesting potential future treatment option for dogs with PDH. In **Chapter 5** we investigated whether two peptide compounds, compounds BIM-22776 and BIM-22A299, were potent MC2R antagonists *in vitro* in canine primary adrenocortical cell culture.

The third potential treatment target to inhibit cortisol production, but possibly also to inhibit ACT growth, would be Steroidogenic factor-1 (SF-1). SF-1 is an orphan nuclear receptor that regulates adrenal steroidogenesis, development, and growth. Increased SF-1 activity through ACTH signaling is an important driver of increased cortisol production in dogs with PDH, and high SF-1 expression in an ACT is related to increased recurrence rates after surgery. Inhibition of SF-1 activity would therefore be an interesting treatment approach for dogs with Cushing's syndrome. The aim of **Chapter 6** was to investigate the effects of three SF-1 inverse agonists, compounds IsoQ A, #31, and #32, on cortisol production, mRNA expression of SF-1 target genes, and cell viability in canine primary adrenocortical cells of both normal adrenals and ACTs.

After a dog with a cortisol-secreting ACT has undergone adrenalectomy, reliable prognostic factors could help to select dogs with high risk of recurrence that might benefit from adjuvant therapy after surgery. Assessing the risk of recurrence is usually based on histopathology. However, whether the histopathological parameters that are often used are really associated with survival times was not previously assessed. Moreover, these histopathological parameters are known to have high interobserver variability in the evaluation of human ACTs. The aim of the study in **Chapter 7** was therefore to establish a reliable and easy-to-use histopathological scoring system for canine cortisol-secreting ACTs that can assess the prognosis of dogs after adrenalectomy. Twenty histopathological parameters and the Ki67 proliferation index were evaluated for their intra- and interobserver agreement scores, and the results were related to the clinical outcome with survival analyses.

The results of Chapter 7 could help to select dogs with high risk of recurrence that could benefit from adjuvant therapy after surgery, for example with mitotane or radiotherapy, to increase their survival times. Additionally, if molecular markers that are associated with malignancy and therefore high risk of recurrence could be identified, these markers could potentially be targets for future treatment approaches. The aim of the study in **Chapter 8** was to investigate the mRNA expression of 14 candidate genes associated with potential druggable targets, and to assess whether their expression was related to the histopathological scoring system described in Chapter 7, and/or with the dogs' survival times.

The results, implications, limitations, and future perspectives of the studies included in this thesis are discussed in **Chapter 9**.

In summary, the aims of this thesis were:

1. To determine which enzymes are involved in canine steroidogenesis, as to discover which enzymes can be used as targets for selective cortisol inhibition (**Chapter 3**)
2. To test the efficacy of potential future treatment options *in vitro* in a canine primary adrenocortical cell culture model (**Chapters 4, 5** and **6**)
3. To identify prognostic indicators in canine cortisol-secreting ACTs, which can help to select dogs with high risk of recurrence after adrenalectomy that could benefit from adjuvant therapy, and which could help to identify potential future treatment targets (**Chapters 7** and **8**)

Chapter

2

General Introduction

Part of this chapter has been published as:

Treating Canine Cushing's Syndrome: Current Options and Future Prospects

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The adrenal glands are paired endocrine organs that are located craniomedial to the kidneys. Each adrenal gland is composed of two functionally and embryologically distinct tissue types: the adrenal medulla and the adrenal cortex. The adrenal medulla produces catecholamines such as epinephrine and norepinephrine, which play an important role in the fight-or-flight response. The adrenal cortex produces different classes of steroid hormones, which control electrolyte metabolism and response to stress. The adrenal cortex consists of three concentric zones: the zona glomerulosa (zG), the zona fasciculata (zF), and the zona reticularis (zR) (Fig. 1).[1,2]

The zG is the outer zone, which lies adjacent to the surrounding capsule. It produces mineralocorticoids, of which aldosterone is the most important one. Aldosterone regulates sodium retention in the kidney and subsequently water retention and blood pressure. Its secretion is regulated by the renin-angiotensin system and by the plasma potassium level.[1,3]

The zF is the middle zone, which lies between the zG and zR. It produces glucocorticoids, of which cortisol is the most important one. Cortisol influences a wide range of body functions, such as carbohydrate, muscle, lipid and bone metabolism, and the immune system. Its secretion is regulated by the hypothalamic-pituitary-adrenal (HPA) axis (see below).[1,3]

The zR is the inner zone, which lies adjacent to the medulla. It produces androgens such as dehydroepiandrosterone (DHEA) and androstenedione.[4,5] The exact role and regulation of adrenal androgen production in dogs is unclear, but the zR increases in size during prepuberal to adult development which could be correlated with sexual maturation.[6]

This zonation pattern holds true for the canine and human adrenal cortex.[1,7] In contrast, the adrenal cortex of adult mice and rats contains no recognizable zR. In the adrenal cortex of young mice, a different layer is present between the zF and medulla, known as the X-zone. In the adrenal cortex of rats, a layer is present between the zG and zF, known as the undifferentiated zone. In the adrenal cortex of ferrets, a similar layer is present between the zG and zF, known as the intermediate zone.[7]

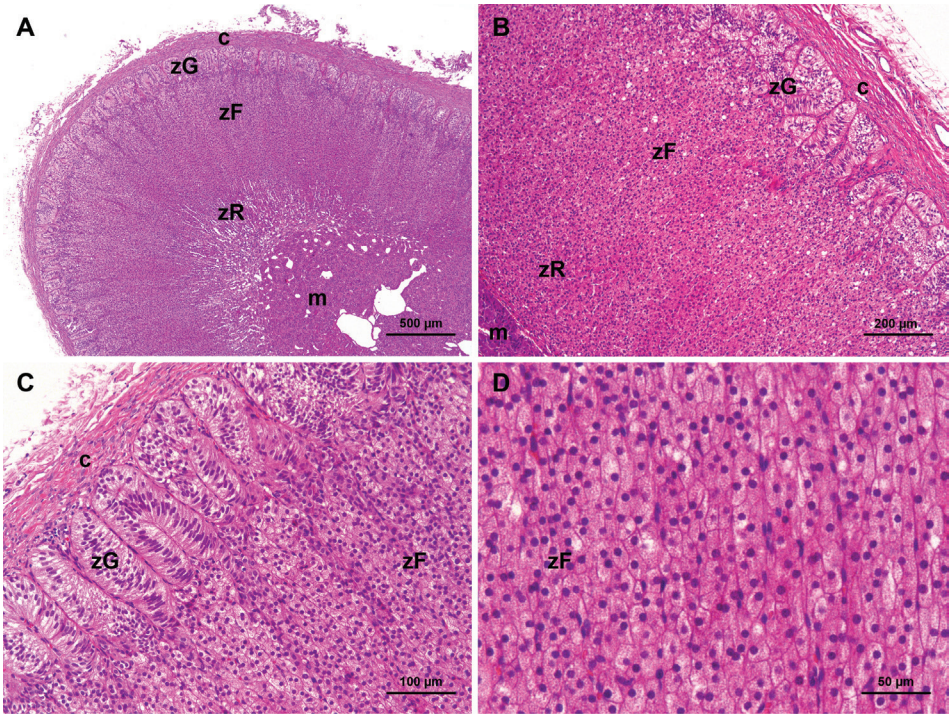

Figure 1 Histology of the canine adrenal gland, hematoxylin and eosin stain. Scale bars: (A) 500 µm; (B) 200 µm; (C) 100 µm; and (D) 50 µm. Abbreviations: c, capsule; zG, zona glomerulosa; zF, zona fasciculata; zR, zona reticularis; m, medulla.

Each steroid hormone is synthesized from a common precursor: cholesterol. Cholesterol is converted to the different steroid hormones in multiple intermediate steps, which are all catalyzed by steroidogenic enzymes.[8] To ensure that each zone in the adrenal cortex produces a different hormone, each zone expresses a different set of steroidogenic enzymes. In humans, zone-specific differences include the presence of aldosterone synthase (CYP11B2) in the zG, which is required for aldosterone production; the presence of 17α-hydroxylase/17,20-lyase (CYP17A1) in the zF and zR, which is required for cortisol and androgen production; and the presence of cytochrome b5 (CYB5) in the zR, which enhances the 17,20-lyase activity of CYP17A1 that is required for androgen production (Fig. 2).[7,8] The adrenal cortex of adult mice and rats lacks CYP17A1 expression, which makes them unable to produce cortisol or adrenal androgens. Adult mice and rats therefore produce corticosterone as the principal glucocorticoid.[7] Because the end-products of the mineralocorticoid and glucocorticoid pathways are the same in dogs as in humans, most textbooks copy the human steroidogenic pathway when talking about canine steroidogenesis.[1,9]

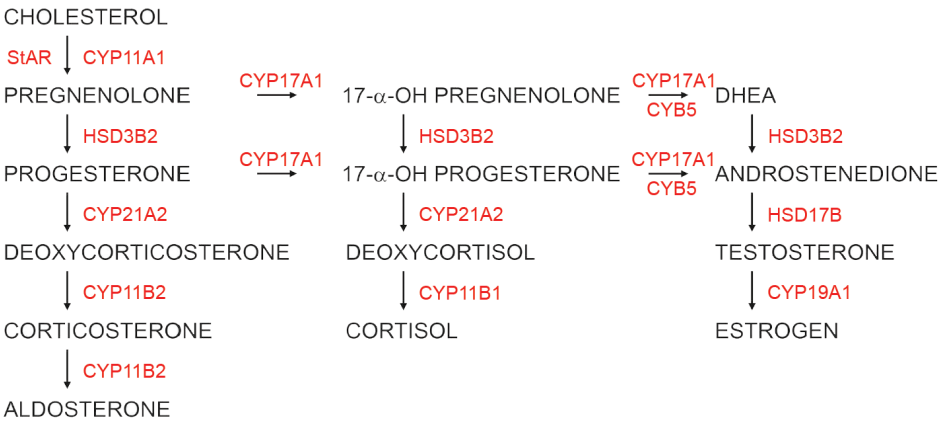

Figure 2 Human steroidogenesis in the adrenal cortex and gonads. Steroid hormones are indicated in black and steroidogenic enzymes and StAR are indicated in red. Abbreviations: StAR, steroidogenic acute regulatory protein; CYP11A1, cytochrome P450 side chain cleavage; HSD3B2, 3β-hydroxysteroid dehydrogenase; CYP17A1, 17α-hydroxylase/17,20-lyase; CYP21A2, 21-hydroxylase; CYP11B2, aldosterone synthase; CYP11B1, 11β-hydroxylase; CYB5, cytochrome b5; HSD17B, 17β-hydroxysteroid dehydrogenase; CYP19A1, aromatase. Figure adapted from Pihlajoki et al. (2015).[7]

Stress response

The stress response serves an important physiological purpose. In response to a stressor, behavioral and physical changes occur, including heightened awareness and redirection of energy to where it is needed.[10] An important part of the stress response is the HPA axis (Fig. 3). Stress stimulates the secretion of corticotropin-releasing hormone (CRH) and arginine-vasopressin (AVP) by the hypothalamus. This causes the precursor pro-opiomelanocortin (POMC) to be cleaved into adrenocorticotrophic hormone (ACTH), which is then secreted from the anterior pituitary.[11] ACTH subsequently binds to the $G_{s\alpha}$-protein-coupled melanocortin 2 receptor (MC2R) in the adrenal cortex, assisted by the melanocortin 2 receptor accessory protein (MRAP), which activates the cyclic AMP (cAMP)-protein kinase A signaling pathway.[12] This signaling pathway eventually results in increased cortisol production, which in turn exerts negative feedback on both the hypothalamus and pituitary gland.[1]

The increased cortisol production in response to ACTH can be divided in an acute phase, which occurs within seconds to minutes, and a chronic phase, which occurs within hours to days. In the acute phase, ACTH binding activates steroidogenic acute regulatory protein (StAR). StAR then mediates transfer of free cholesterol from the outer to the inner mitochondrial membrane, which initiates steroidogenesis. In the chronic phase, ACTH binding increases the expression of steroidogenic enzymes and StAR, and genes that are involved in cholesterol availability.[12] This effect on gene expression is mediated by several transcription factors, of which one of the most important is the orphan nuclear receptor Steroidogenic factor-1 (SF-1). SF-1 is not only important in steroidogenesis, but also an essential factor for adrenocortical development.[13]

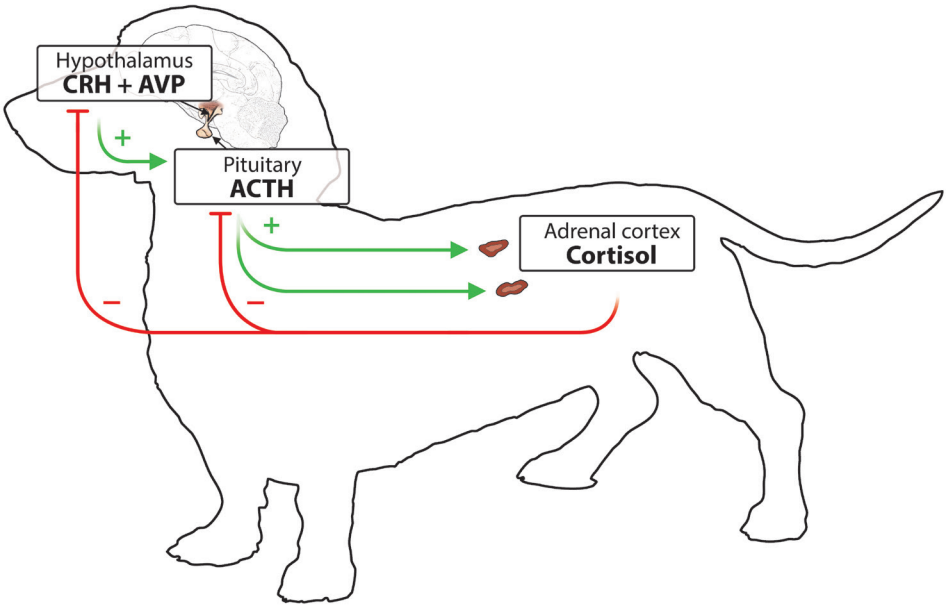

Figure 3 Schematic illustration of the hypothalamic-pituitary-adrenal axis. The hypothalamus secretes CRH and AVP, which stimulate ACTH secretion by the pituitary gland, which stimulates cortisol secretion by the adrenal cortex. Cortisol then inhibits its own production via a negative feedback loop involving both the hypothalamus and pituitary. Abbreviations: CRH, corticotropin-releasing hormone; AVP, arginine-vasopressin; ACTH, adrenocorticotrophic hormone.

In healthy individuals, the stress system is a complex but well-regulated network that is helpful in stressful situations.[10] However, this network can get deregulated, as is the case in Cushing's syndrome.

Cushing's syndrome

Hypercortisolism, often referred to as Cushing's syndrome, was described by the neurosurgeon Harvey Cushing in 1932,[14] and is characterized by chronically increased circulating glucocorticoids. Hypercortisolism can be either iatrogenic, caused by glucocorticoid administration, or occur naturally, caused by excessive endogenous cortisol production.[1] Naturally occurring hypercortisolism is a common endocrine disorder in dogs, with an incidence of 1–2 cases per 1000 dogs per year.[15,16] In 80–85% of cases, the condition is caused by an ACTH-secreting pituitary adenoma (pituitary-dependent hypercortisolism; PDH). In the remaining 15–20%, it is most often caused by a cortisol-secreting adrenocortical tumor (ACT).[1] Rare causes of hypercortisolism in dogs include ectopic ACTH syndrome[17] and food-dependent hypercortisolism.[18]

Diagnosis

The diagnosis of hypercortisolism should be based on the dog's medical history and clinical signs. Hypercortisolism usually occurs in middle-aged to older dogs.[16,19] The most common clinical signs include polyuria, polydipsia, polyphagia, central obesity, hepatomegaly, panting, muscle atrophy, progressive bilateral alopecia, and systemic hypertension (Fig. 4). Other clinical signs include hyperpigmentation, calcinosis cutis and insulin-resistant diabetes mellitus.[1,16,20] Additionally, the pituitary tumor or ACT can induce mass-occupying effects. In cases with a large pituitary tumor, these effects include neurological signs such as anorexia, lethargy, and altered behavior. In cases with an ACT, these effects develop secondary to metastases or invasion of the ACT into the phrenicoabdominal vein or caudal vena cava.[1,20]

When there is clinical suspicion of hypercortisolism, the results of a complete blood count, serum biochemistry panel, and urinalysis may further support the diagnosis. Abnormalities that can be found in these tests include the presence of a stress leukogram, increased serum alkaline phosphatase activity, and low urine specific gravity. None of these findings are pathognomonic, but can be supportive of hypercortisolism.[20]

Endocrine tests should be used to further confirm the suspicion of hypercortisolism. It is important to only test for hypercortisolism in dogs with a high degree of clinical suspicion to decrease the chance of false-positive results.[21] The recommended screening tests are the low-dose dexamethasone suppression test or the urinary corticoid:creatinine ratio (UCCR). The UCCR can also be combined with the high-dose dexamethasone suppression test (HDDST). When the hypercortisolism is suppressible (>50%) by dexamethasone the dog is diagnosed with PDH. When the hypercortisolism is non-suppressible, further differentiation requires measurement of plasma ACTH concentration and diagnostic imaging. A CT or MRI scan is preferred to determine the size and contour of the pituitary and adrenal glands, and in case of an ACT also to detect vascular invasion and to screen for metastases.[1,19,20] Moreover, pituitary tumors and ACTs can coexist,[22,23] which could be missed without complete imaging. Differentiating between the two main causes of hypercortisolism is essential when choosing the optimal treatment strategy.[20]

Comparative pathobiology

Many similarities exist between hypercortisolism in dogs and humans, including the etiology, clinical signs, diagnostics, and medical care.[24,25] Moreover, adrenocortical zonation and the end-product of the glucocorticoid pathway is comparable between the two species. Consequently, new insights in human hypercortisolism can advance the understanding of and treatment for canine hypercortisolism, and vice versa. In this chapter we will therefore not only focus on current treatment options for canine hypercortisolism, but also on advancements in the treatment of human hypercortisolism. Additionally, we discuss promising drugs that might develop into future treatment options.

Figure 4 A ten year old male dachshund with typical signs of hypercortisolism: central obesity, alopecia, and muscle atrophy.

Therapy

The goals of treating canine hypercortisolism would optimally be to eliminate the source of either ACTH or autonomous cortisol excess, to achieve normocortisolism, to eliminate the clinical signs, to reduce long-term complications and mortality, and to improve the quality of life. Surgical removal of the causal tumor or radiotherapy are currently the only treatment options that have the potential to eliminate the source of either ACTH or autonomous cortisol excess. However, these options are not without risks, not widely available and not appropriate for every patient. Pharmacotherapy is a commonly used treatment that aims to eliminate the clinical signs of the condition. A combination therapy of medical treatment with radiotherapy is also possible.[1,26]

Without treatment, dogs with PDH have a median survival time of 359 days (95% confidence interval (CI), 271–829)[27] to 506 days (95% CI, 292–564).[28] There are no data on the survival of dogs with a cortisol-secreting ACT without treatment.

Surgery

Hypophysectomy

Hypophysectomy in dogs is performed using a transsphenoidal approach where the entire pituitary gland is removed.[29,30] In a recent study with a large cohort of 306 dogs with PDH that underwent hypophysectomy, 91% of the dogs were alive after 4 weeks, of which remission was confirmed in 92%.[31] Of the dogs that were in remission, disease recurrence was observed in 27%. The median survival time was 781 days (range, 0–3808 days) and the median disease-free interval of the dogs that were in remission was 951 days (range, 31–3808 days).[31]

Replacement therapy after hypophysectomy consists of life-long administration of glucocorticoids and thyroxine, and temporary administration of desmopressin, a synthetic vasopressin analogue.[1,29,32] The main complications of hypophysectomy are perioperative death, transient mild postoperative hypernatremia, transient reduction or cessation of tear production, prolonged or permanent diabetes insipidus, and recurrence of hypercortisolism.[29,30]

Factors that negatively influence the prognosis include a high pituitary height/brain area (P/B) value, old age, high preoperative circulating ACTH concentration, and high pre- and postoperative UCCRs.[31,33,34] Although a high P/B value is a negative prognostic indicator, hypophysectomy remains a good treatment option also for large pituitary tumors.[31,35] The main limitation of hypophysectomy is that it is available only in large veterinary centers with an established team of experienced surgeon(s), anesthetist(s), critical care specialist(s) and endocrinologist(s), with consequently high initial costs.[26]

Adrenalectomy

Adrenalectomy is recommended for dogs with uni- or bilateral ACT. Adrenalectomies were traditionally performed as ventral or paracostal open laparotomies. Perioperative mortality rates were quite high in initial studies,[36] but improved in later studies[37–41] and are as low as 6–8% in most recent studies.[42,43] Adrenalectomy can also be performed laparoscopically. Laparoscopic adrenalectomy has been used in human medicine since the early 1990s and has recently been gaining interest and shown to have benefits in veterinary medicine as well.[43,44]

Reported median survival times for dogs undergoing adrenalectomy range from 778 days (range, 1–1593)[38] to 953 days (range, 0–1941).[41] When dogs survive the perioperative period, the long-term survival is good.[38,42] The main complications that can occur include minor to severe hemorrhage, hypotension, tachycardia and peri-operative death.[37,41–43] The tumor capsule can rupture, possibly more often in laparoscopic than in open adrenalectomies, but does not commonly lead to tumor regrowth.[43] The main complications that can occur postoperatively include pancreatitis and thromboembolism.[37,38,43] The reported

hypercortisolism recurrence rate varies between 12%[38] and 30%,[37] which can be either because of regrowth of the ACT or metastases.

Adrenalectomy is not recommended in patients that have metastases or extensive vascular invasion, which is why thorough presurgical diagnostic imaging is imperative. Vascular invasion does not necessarily exclude patients from undergoing adrenalectomy, since some studies indicate that tumor invasion in the caudal vena cava does not affect perioperative mortality,[39,42] and techniques to remove the tumor thrombus have improved.[45] However, when the vascular invasion is extensive, in particular when the tumor invasion in the vena cava extends beyond the hepatic hilus, the perioperative mortality rates can increase.[46] Interestingly, when patients with vascular invasion survive the perioperative period, their long-term survival is not worse than that of patients without vascular invasion.[42,46]

In humans with adrenocortical carcinomas (ACCs), patients with high risk of recurrence often receive some form of adjuvant treatment after adrenalectomy to improve their survival times.[47] In dogs, however, little is known about which factors indicate that the dog has a high risk of recurrence. Identifying prognostic markers in dogs with cortisol-secreting ACTs would greatly assist the selection of dogs with high risk of recurrence that could benefit from adjuvant treatment after adrenalectomy.

Radiotherapy

Pituitary radiotherapy

Radiotherapy (RT) can be useful to decrease tumor size and reduce neurological signs in large pituitary tumors.[9,26,27] Usually, a total dose of 36–48 Gy is administered in 3–4 Gy fractions, which requires the dog to be under anesthesia on approximately twelve occasions. The tumor size decreases after RT in most cases, but the time to effect and whether it diminishes the clinical signs of hypercortisolism can vary considerably between patients.[27,48–50] Temporary or permanent additional pharmacotherapy may therefore be required to manage hypercortisolism. Adverse effects that can occur after RT are pituitary hemorrhage and otitis media.[50] The median (±SD) survival time was 539 days (±51) in one study,[49] and was not reached in a study with a median follow-up time of 702 days (range, 27–1927).[27]

In humans with PDH, radiotherapy is currently primarily applied as single-session, focused stereotactic radiosurgery (SRS) using a Gamma Knife, where image-guided precisely-targeted radiation is applied at high-dose fractions. The use of SRS is well tolerated in humans and may also result in shorter response times than conventional RT.[51] The same technique has recently been applied to dogs with pituitary tumors and showed promising results.[52,53]

Adrenal radiotherapy

Information on the use of RT in canine ACTs is limited: only one study on RT in canine ACTs has been published so far,[54] in which nine dogs with ACTs with vascular invasion were enrolled. The ACTs showed progressive shrinkage in varying degrees in all dogs, consistent with a partial response.[54] More research is required to determine the efficacy of RT in dogs with ACTs.

In humans, RT is sometimes used for postoperative treatment of ACCs. Although earlier publications reported that human ACCs are resistant to RT, more recent studies show that RT can improve local tumor control.[55–57] Postoperative RT could potentially be useful for dogs with high risk of recurrence after adrenalectomy.

Pharmacotherapy – adrenal-targeting drugs

Pharmacotherapy is often used to control the clinical signs of hypercortisolism. Trilostane is the drug of choice for dogs with PDH, and in case of an ACT either trilostane or mitotane can be used.

Trilostane

Trilostane is a synthetic steroid analogue that competitively inhibits the steroidogenic enzyme 3β-hydroxysteroid dehydrogenase (HSD3B2),[58] which is required for the production of all classes of adrenocortical hormones (Fig. 5). Trilostane therefore inhibits both cortisol production, which results in a loss of negative feedback and a compensatory increase in plasma ACTH concentration,[59] and aldosterone production, which causes a compensatory increase in plasma renin activity.[60,61] Additionally, trilostane possibly also inhibits other enzymes in the steroidogenesis cascade, such as 11β-hydroxylase (CYP11B1).[62,63]

Trilostane is registered for the medical management of both canine PDH and cortisol-secreting ACTs, but most studies on the use of trilostane have been performed in dogs with PDH. Trilostane is absorbed rapidly from the gastrointestinal tract. Because administration with food significantly increases the rate and extent of absorption, trilostane should always be given with food.[64]

There is a marked variation in the optimal trilostane dose, and the current recommendations are to start with much lower dosages than originally recommended by the manufacturer, which can be equally effective but induce fewer adverse effects than higher dosages.[65–67] Larger dogs generally need a lower dose per kg body weight than smaller dogs.[66] Because the duration of cortisol suppression is less than 12 h in most dogs, administrating trilostane twice daily can improve the clinical response while keeping the total daily dose relatively low, and significantly reducing the adverse effects.[65,68–71] The authors of this review advise to start treatment for PDH with an initial dose of 0.5–1 mg/kg twice daily. If twice daily treatment is undesirable for financial or practical reasons, the initial dose should be 1–2 mg/kg once daily.

Within weeks, an adequate dose of trilostane can increase the dog's activity and reduce polyuria, polydipsia and polyphagia. More time is needed to observe notable improvements on the skin and hair coat, which can take months. The hair coat can sometimes initially appear to worsen, due to shedding of telogen hairs and dry skin scales.[26]

Trilostane is usually well tolerated, but the main adverse effect that can occur is transient hypocortisolism (shortage of glucocorticoids), possibly combined with or followed by complete hypoadrenocorticism (shortage of both glucocorticoids and mineralocorticoids). A recent study reported that the chance of a dog having at least one episode of clinical hypocortisolism within the first 2 years of trilostane treatment is approximately 15%.[71] In most dogs, the adverse effects resolve once trilostane treatment is withdrawn. In such cases continuation of treatment with a lower dose is recommended when clinical signs of hypercortisolism recur. However, in some dogs, the hypoadrenocorticism can be permanent, which is possibly the result of adrenal necrosis, and can be fatal in severe cases.[71–73] One study found at postmortem examinations that adrenal necrosis was present in four out of six dogs with PDH that were treated with trilostane.[74] Subsequent studies suggested that it is not trilostane but rather the increased ACTH production resulting from a loss of negative feedback that causes this adrenal necrosis.[60,75] The reported median survival times of dogs with PDH treated with trilostane range from 662 days (range, 8–1971)[76] to 852 days (range, 2–3210).[77]

For successful management of PDH with trilostane, frequent monitoring is essential. In the last decade efforts have been made to identify the best method to monitor trilostane therapy. In all methods, evaluation of the clinical signs is the first step. The preferred monitoring method is the use of the ACTH-stimulation test, which monitors the adrenal glands' reserve capacity to secrete cortisol.[78,79] The timing of the ACTH-stimulation test is crucial since this influences the results,[80] and the recommendation is to coincide the test with the maximal trilostane action (2–4 h after trilostane administration).[81] Despite its widespread use, the ACTH stimulation test has never been validated as a monitoring tool for trilostane therapy, and there are some concerns regarding the variation in results depending on the timing of the test and whether this reflects clinical control.[82,83] Moreover, tetracosactide (synthetic ACTH [1–24]) is not easily available in all countries. A recently proposed alternative method is to measure the pre-pill cortisol (Pre-Vetoryl Cortisol; PVC) concentration and compare it to the clinical signs reported by owners. The PVC was found to better reflect the clinical control than the ACTH stimulation test.[83] However, even dogs with excellent clinical control can have insufficient adrenocortical reserve capacity, which can become clinically relevant when they face stress situations.[71] The PVC approach is not comparable to the principle of the ACTH stimulation test: it is not a measure of the adrenocortical reserve and will therefore not reflect the safety of trilostane therapy. The applicability of this alternative method will have to be determined in future studies.

In dogs with PDH, trilostane effectively controls the clinical signs of glucocorticoid excess but does not directly affect the growth of the pituitary tumor. This is irrelevant initially in dogs with non-enlarged pituitary glands (i.e., P/B value ≤ 0.31 mm/mm^2),[31] but the pituitary tumor might grow over time. In healthy dogs, the P/B value has been shown to significantly increase following trilostane therapy,[84] but this has not been studied in dogs with PDH. Nonetheless, especially in younger dogs, a control CT or MRI scan after 1 year of treatment could be considered to re-evaluate the pituitary size.

In dogs with a cortisol-secreting ACT it is important to remember that while these tumors are mostly malignant, trilostane will only reduce the clinical signs and not affect the growth or possible metastases of the ACT. Palliative treatment with trilostane has been shown to be effective in controlling clinical signs.[1,85,86] The reported median survival times of dogs with ACTs treated with trilostane range from 353 days (95% CI, 95–528)[87] to 427 days (range, 101–1678).[88] Although there is no scientific data available to support this, it is the authors' experience that dogs with an ACT can be more sensitive to trilostane treatment, which is why the authors advise to start the treatment with a relatively low initial dose of 0.5 mg/kg twice daily.

Mitotane

Mitotane (o,p'-DDD) is an adrenocorticolytic agent that leads to progressive adrenocortical necrosis and atrophy. Mitotane also inhibits the steroidogenic enzymes cytochrome P450 cholesterol side-chain cleavage enzyme (CYP11A1) and CYP11B1 (Fig. 5), which contributes to inhibition of cortisol synthesis,[89,90] and induces other cytochrome P450 enzymes such as cytochrome P450 enzyme 3A4 (CYP3A4), which leads to increased metabolic clearance of glucocorticoids.[91]

Although mitotane has been used to treat hypercortisolism for decades, the exact mechanism of action was poorly understood. A recent study found that one of its mechanisms of action is inhibition of sterol-O-acyl-transferase 1 (SOAT1), an enzyme that catalyzes the conversion of free cholesterol to cholesterol esters. Inhibition of this conversion increases the amount of free cholesterol in the cell, which can lead to endoplasmic reticulum stress and, subsequently, cell apoptosis.[92] Interestingly, the dog is much more sensitive to mitotane than other species,[93] which makes the dog an interesting candidate to further elucidate the mechanism of action.

The use of mitotane for the treatment of canine PDH has largely been replaced by that of trilostane.[1] This is mostly because trilostane is just as effective, is safer to handle and has been associated with fewer adverse effects than mitotane.[64,94] However, in case of an ACT, treatment with mitotane is still a good option because it has the added advantage that it can destroy ACT cells.

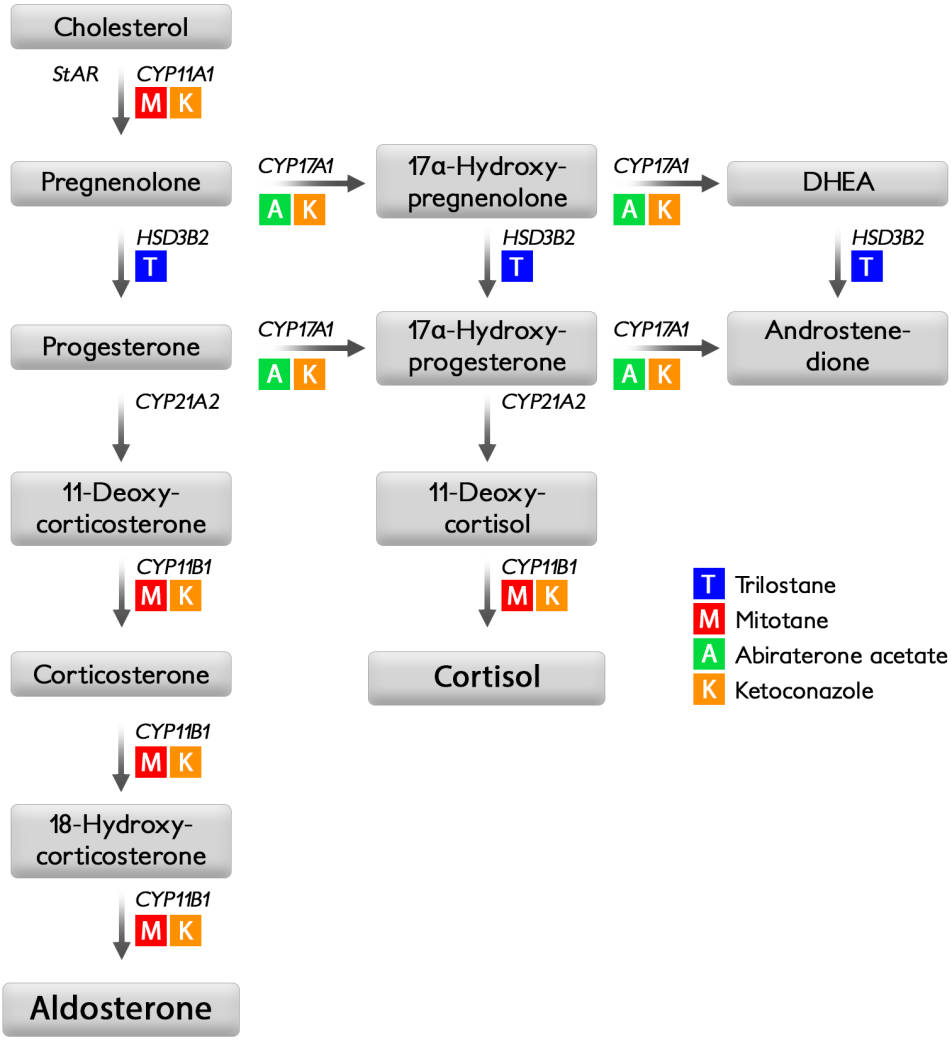

Figure 5 Schematic overview of the site of action of steroidogenesis inhibitors. Abbreviations: StAR, steroidogenic acute regulatory protein; CYP11A1, cytochrome P450 side chain cleavage; HSD3B2, 3β-hydroxysteroid dehydrogenase; CYP17A1, 17α-hydroxylase/17,20-lyase; CYP21A2, 21-hydroxylase; CYP11B1, 11β-hydroxylase.

Because the goal of mitotane therapy in cases with an ACT is not only to reduce cortisol production but also to destroy as many neoplastic cells as possible, a non-selective protocol that affects the entire adrenal cortex should be considered.[1,95] This treatment protocol consists of 50 to 75 mg/kg mitotane per day: daily for 5 days and then every other day over 40 days. For dogs of small breeds, a higher dose of up to 100 mg/kg may be required. Each daily dose should be divided into three or four portions.[1,96] For sufficient absorption, mitotane should be given with food.[97] Substitution therapy starts at the third day and consists of daily glucocorticoids (e.g. 2 mg/kg cortisone acetate), mineralocorticoids (e.g. 0.0125

mg/kg fludrocortisone acetate) and salt (0.1 g/kg sodium chloride), all divided in at least two portions.[1] After the initial course of mitotane has been administered, the glucocorticoid dose is reduced (e.g. 0.5–1 mg/kg cortisone acetate), but doubled for one or two days in the event of injury, severe physical stress, or anesthesia. To prevent recurrence, mitotane should be administered at the initially daily dose once weekly for at least 6 months, or even lifelong.[1]

Adverse effects of mitotane include anorexia, lethargy, weakness and diarrhea.[95] If the dog develops adverse effects, the mitotane treatment has to be temporarily discontinued, but not the substitution therapy. If these adverse effects are ignored and the owner continues to give mitotane, this can result in a potentially fatal hypoadrenocorticoid crisis (particularly if they continue to vomit or refuse substitution therapy). When the owner is given clear instructions this rarely occurs and the mitotane administration can usually be resumed after a few days.[1] Due to the cytotoxicity of mitotane, it should not be used when there are young children or pregnant women in the household.[1]

To evaluate whether the ACT has been completely destroyed, the UCCR can be measured in morning urine after the initial course of mitotane (i.e., after day 45) and every 6 months thereafter. Prior to morning urine collection for this UCCR measurement, the evening doses of glucocorticoids and mineralocorticoids should be withheld. Complete destruction of the ACT results in very low or even undetectable UCCRs. In addition to UCCR measurements, regular blood sodium and potassium measurements are required, which help to regulate the dose of mineralocorticoids that are administered.[1] The reported median survival times of dogs with ACTs treated with mitotane range from 102 days (95% CI, 43–277)[87] to 476 days (range, 61–1129)[88] and did not differ significantly from the survival times for dogs treated with trilostane in both studies. However, these studies did not use the mitotane protocol as described herein (aimed at complete adrenocortical destruction), which might give different results.

In humans, mitotane is used in patients with nonresectable ACCs, and as adjuvant therapy after surgical resection of an ACC, especially in patients with a high recurrence risk. In the patients with nonresectable ACCs, and in the patients with local or metastatic recurrence, approximately 25–30% responds to mitotane treatment.[92,98] Since mitotane was shown to be a SOAT1 inhibitor, the degree of response could depend on SOAT1 expression. Indeed, Sbiera *et al.* (2015) showed that human ACCs with high SOAT1 expression responded better to mitotane treatment than those with low SOAT1 expression.[92] A chemotherapy protocol where mitotane was combined with etoposide, doxorubicin and cisplatin seemed to give the best results in terms of progression-free survival times.[99] Prognostic indicators could be helpful, as well as determination of the SOAT1 expression, to select dogs with a high recurrence risk that might benefit from adjuvant mitotane therapy following adrenalectomy. Although more research is required, the SOAT1 expression might provide insight into whether the ACT or its metastases will respond to mitotane.

Ketoconazole

Ketoconazole is a synthetic imidazole derivative which was originally developed as an antifungal agent. Ketoconazole inhibits multiple cytochrome P450 enzymes, including CYP11A1, CYP17A1 and CYP11B1 (Fig. 5),[100] thereby resulting in inhibition of cortisol production. Ketoconazole has been used for the treatment of hypercortisolism in dogs, but the percentage of non-responders was relatively large (approximately 25%) and it caused more adverse effects than trilostane.[1,9]

Levoketoconazole is an enantiomer of ketoconazole that has been purified from racemic ketoconazole, and has been reported to be a more potent inhibitor of cortisol production with reduced hepatotoxicity as compared to ketoconazole *in vitro* and *in vivo* in humans.[101] It is currently under development in a phase III clinical trial in humans.[101,102]

Future prospects – adrenal-targeting drugs

The main downsides of current medical adrenal-targeting treatment options include low selectivity for the glucocorticoid pathway, the possibility of overdosage, and occurrence of disruptive changes in the adrenal cortex, which necessitate careful dosing schemes and regular check-ups. In this section we provide an overview of interesting candidates that might prove to have superior selectivity, effectivity and/or tolerability when compared to currently available treatment options.

Melanocortin 2 receptor antagonists

The MC2R is the receptor for ACTH and is expressed only in the adrenal cortex. It is a member of the melanocortin receptor subfamily of type 1 $G_{s\alpha}$-protein-coupled receptors, and is highly selective for ACTH.[103] A selective antagonist of the MC2R could therefore have great potential in the medical treatment of PDH, since it would directly block the excessive ACTH stimulation without having other (intra-adrenal) effects. The selectivity of an MC2R antagonist for the MC2R is crucial, since inadvertently antagonizing or agonizing any of the other melanocortin receptors could result in multiple adverse effects.[104,105] A recent study showed that two ACTH analogs, GPS1573 and GPS1574, are potent antagonists of the MC2R in vitro. However, these peptides also had some agonistic and/or antagonistic effects on other melanocortin receptors, and subsequent studies in rats showed disappointing results *in vivo*.[106,107] Further developments might eventually generate a selective MC2R antagonist that could be used as a medical treatment option in dogs with PDH.

Abiraterone acetate

In humans, CYP17A1 is required for cortisol but not for aldosterone production. Selective inhibition of CYP17A1 could therefore be an interesting treatment approach in hypercortisolism, since this would inhibit the production of cortisol but not that of aldosterone. One known CYP17A1 inhibitor is abiraterone acetate (Fig. 5), which is approved in the

USA for use in human patients with castration-resistant prostate cancer to inhibit androgen (precursor) production by the adrenal glands.[108] Abiraterone acetate also inadvertently induced hypocortisolism in these patients.[109] To determine whether AA has potential as a treatment option for canine hypercortisolism, more studies on the steroidogenic enzyme expression in the canine adrenal cortex are required.

Steroidogenic factor-1 inverse agonists

SF-1 is an orphan nuclear receptor that regulates adrenal development, growth, and steroidogenesis.[110] ACTH stimulates the transcriptional activity of SF-1, which increases the transcription of genes that encode steroidogenic enzymes.[111] Increased SF-1 activity is therefore an important characteristic in dogs with PDH. Moreover, earlier work by our group showed that *SF-1* mRNA expression was significantly higher in ACTs of dogs in which the hypercortisolism recurred within 2.5 years after adrenalectomy, than in ACTs of dogs without recurrence.[112] Inhibition of SF-1 activity could therefore be a potential treatment approach in both PDH and ACTs.

Sterol-O-acyl-transferase 1 inhibitors

As mentioned previously, one recently discovered mechanism of action of mitotane is that it inhibits SOAT1 and thereby increases the amount of free cholesterol, which is toxic for the cell. More selective SOAT1 inhibitors that have the same adrenocorticolytic effects as mitotane but with fewer off-target adverse effects could possibly be interesting for a targeted treatment approach in dogs with non-operable or metastasized ACTs. In the early 1990s, researchers described that ATR-101, a SOAT1 inhibitor, potently induced selective adrenocorticolysis in healthy beagle dogs.[113] ATR-101 recently regained interest and is currently being studied as a possible future treatment for humans with ACCs.[114–116] Because the effect of ATR-101 is particularly apparent in dogs,[117] this could be an interesting treatment approach in dogs with ACCs.

Pharmacotherapy – pituitary-targeting drugs

Medical management of PDH would ideally target the pituitary tumor. Because dopamine (DA) and somatostatin (SST) both have inhibitory functions in the pituitary gland, the main focus in research on pituitary-targeting drugs are three receptor subtypes: DA receptor subtype 2 (DRD2), and SST receptors subtype 2 (SSTR2) and subtype 5 (SSTR5). In canine corticotroph adenomas, the receptor subtype that is mainly expressed is SSTR2, while DRD2 and particularly SSTR5 are expressed at much lower levels.[118] When comparing treatments between dogs and humans, it's important to realize that this distribution is somewhat different in human corticotroph adenomas, where the main receptors are SSTR5 and DRD2.[118] There are currently no pituitary-targeted drugs that are registered for use in canine PDH.

Cabergoline

Cabergoline is a DA agonist that binds to the DRD2. In line with the moderate DRD2 expression in canine corticotroph adenomas, canine corticotroph cells responded only modestly to cabergoline *in vitro*.[118] However, *in vivo* experiments showed that 43% of dogs with PDH responded well to treatment with cabergoline, with fewer clinical signs, smaller pituitary adenomas and lower UCCRs.[119] This observed difference in efficacy could possibly be explained by the different durations of treatment.[118]

Pasireotide

Pasireotide (SOM230) is a multiligand SST analog that binds to the SST receptors 1, 2, 3 and 5.[120] In 20 dogs with PDH, pasireotide decreased the plasma ACTH concentration and improved the clinical signs, while no severe adverse effects were observed.[121] In a recent study, pasireotide was administered to dogs with macroadenomas that were also treated with trilostane or mitotane. The pituitary tumor volume decreased in six out of nine dogs, and increased in the remaining three, while no neurologic signs or grossly apparent adverse effects were observed.[122] In humans, the efficacy of pasireotide has been demonstrated, and it has been approved both in Europe and in the USA for the second-line treatment of patients with PDH when surgery has failed or is not an option.[123,124] Due to their expression profile, a SST analog that has higher affinity for the SST2 than pasireotide could prove to be more effective in dogs, as also demonstrated during *in vitro* experiments.[118]

Octreotide

Octreotide is a SST analog that binds to SSTR2 with high affinity, and to SSTR3 and SSTR5 with moderate affinity.[125] In line with the high SSTR2 expression in canine corticotroph adenomas, octreotide significantly inhibited ACTH release in canine corticotroph cells *in vitro*, and did so more effectively than either pasireotide or cabergoline.[118] In humans, octreotide can cause gastrointestinal side effects, but this is less well documented for dogs. For other indications such as insulinoma, octreotide is sometimes used as adjuvant treatment to inhibit insulin secretion. However, its short duration time after subcutaneous injection limits its use.[126,127] Recently, a new technology has been developed that increases the absorption of drug molecules through transient opening of the tight junctions of the gut epithelium, which can achieve therapeutic octreotide levels after oral ingestion in humans.[128] An advantage of oral octreotide could be the lack of injection-related side effects, but there is a need for a strict twice daily fasted dosing regimen in humans.[128] Due to the high SSTR2 expression in canine corticotroph tumors, the availability of oral octreotide treatment could be an interesting treatment approach for dogs with PDH.

Dopamine/somatostatin chimeras

Although the effectivity of individual DA or SST analogs has been proven in the treatment of human pituitary adenomas, a considerable percentage of patients respond poorly or not at all to these treatments. An interesting new approach that is currently being developed

is the use of DA/SST chimeras, which can cause SST and DA receptors to heterodimerize and generate a more effective hybrid receptor.[129] This treatment approach seems very promising, and developments to produce an effective SSTR2/SSTR5/DRD2 chimera are ongoing.[130,131]

Retinoic acid

To produce ACTH, the precursor molecule POMC is cleaved into multiple peptide hormones. The gene expression of POMC is regulated by many factors, including the transcription factors AP-1 and Nur77. Retinoic acid is an agent that regulates multiple cellular processes, including reducing the binding of these transcription factors to their DNA binding sites, ultimately inhibiting ACTH secretion. In 22 dogs treated with retinoic acid, investigators reported a decreased plasma ACTH concentration, decreased UCCR, resolved clinical signs, and decreased pituitary size.[132] In humans, adverse effects such as teratogenicity, mucocutaneous toxicity, defects in liver function and severe photosensitivity have been reported, which might be reduced by limiting the exposure to light.[102] A recent study showed that 9-cis RA, an active isomer of retinoic acid, activates the DRD2 promoter and thereby sensitizes pituitary adenomas for dopaminergic treatments.[133] Additionally, a synthetic retinoid analog named bexarotene has been reported to induce hypopituitarism,[133,134] and a phase I and II clinical trial has been initiated in humans.*

Conclusions

Differentiating between PDH and a cortisol-secreting ACT is essential when choosing the optimal treatment strategy. Surgical removal of the causal tumor is a good option in both cases, or radiotherapy in case of PDH, since these are currently the only treatment options with the potential to eliminate the source of either ACTH or autonomous cortisol excess. However, these options are not without risk, not generally available and not suitable for every patient. Pharmacotherapy is therefore often used, with trilostane advised in dogs with PDH, and either trilostane or mitotane in dogs with an ACT. However, both options have disadvantages. Interesting new drugs have surfaced which could be candidates as future treatment options for canine hypercortisolism. Studies are warranted to test these candidates, for which an *in vitro* cell culture model would provide much pre-clinical information without the requirement of laboratory animals. In case of ACTs, more insight into prognostic markers would help to select dogs with high risk of recurrence that could benefit from adjuvant therapy after adrenalectomy, and could also identify potential treatment targets. A more selective and preferably also tumor-targeted approach could have many advantages for both PDH and ACTs.

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Part

I

Steroidogenesis

Chapter

3

New Insights in the Functional Zonation of the Canine Adrenal Cortex

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3

Abstract

Background: Current understanding of adrenal steroidogenesis is that the production of aldosterone or cortisol depends on the expression of aldosterone synthase (CYP11B2) and 11β-hydroxylase cytochrome P450 (CYP11B1), respectively. However, this has never been studied in dogs, and in some species a single CYP11B catalyzes both cortisol and aldosterone formation. Analysis of the canine genome provides data of a single *CYP11B* gene which is called *CYP11B2*, and a large sequence gap exists near the so-called *CYP11B2* gene. *Objectives:* To investigate the zonal expression of steroidogenic enzymes in the canine adrenal cortex, and to determine whether dogs have one or multiple *CYP11B* genes. *Animals:* Normal adrenal glands from 10 healthy dogs. *Methods:* Zona fasciculata (zF) and zona glomerulosa (zG) tissue was isolated by laser microdissection. The mRNA expression of steroidogenic enzymes and their major regulators was studied with RT-qPCR. Southern blot was performed to determine whether the sequence gap contains a *CYP11B* gene copy. Immunohistochemistry (IHC) was performed for 17α-hydroxylase/17,20-lyase (CYP17A1). *Results:* Equal expression (P = 0.62) of the so-called *CYP11B2* gene was found in the zG and zF. Southern blot revealed a single gene. *CYP17A1* expression (P = 0.05) was significantly higher in the zF compared to the zG, which was confirmed with IHC. *Conclusions and clinical importance:* We conclude that there is only one *CYP11B* gene in canine adrenals. The zone-specific production of aldosterone and cortisol is probably due to zone-specific CYP17A1 expression, which makes it an attractive target for selective inhibition of cortisol synthesis without affecting mineralocorticoid production in the zG.

Introduction

In the canine adrenal cortex, three functional and morphological layers can be distinguished: the outermost zona glomerulosa (zG), the middle zona fasciculata (zF) and the inner zona reticularis (zR).[1,2] The zG is responsible for the production of the mineralocorticoid aldosterone, which is regulated by the plasma potassium, angiotensin-II and ACTH concentration. The zF and zR function together as a unit and are the main source of body's glucocorticoid secretion, which is strictly regulated by the hypothalamic-pituitary-adrenocortical axis. Additionally, the zR may also produce androgens such as dehydroepiandrosterone and androstenedione.

Using cholesterol as a uniform precursor molecule, zone-dependent enzyme expression is essential for distinct steroid production.[1-4] In humans, rats and mice, the last steps in aldosterone and cortisol/corticosterone synthesis are catalyzed by two different but related enzymes: the zG-specific aldosterone synthase (CYP11B2) and zF (and zR)-specific 11β-hydroxylase cytochrome P450 (CYP11B1), respectively (Fig. 1).[5-7] In contrast, cows,[8] pigs,[9] sheep,[10] birds and amphibians[11,12] have only CYP11B1 to catalyze both cortisol and aldosterone formation.

Because humans and dogs have the same adrenocortical end products, i.e. aldosterone and cortisol, human and canine steroidogenesis was assumed to be identical. Therefore, in current veterinary literature, the published cascade of canine adrenocortical steroidogenesis is mainly a human variant.[1,13,14] However, according to the NCBI database, the canine genome does contain a *CYP11B2* (NC_006595.3) gene, but no *CYP11B1* can be found. Near this so-called *CYP11B2* gene on chromosome 13 lies a large sequence gap. The *CYP11B* gene duplicates in humans and mice lie very close to each other,[6] so this sequence gap could be the place for another canine *CYP11B* gene copy. The question is whether the dog has both *CYP11B1* and *CYP11B2*, or if the only canine *CYP11B* gene has erroneously been denominated *CYP11B2*.

In animals with both CYP11B1 and CYP11B2, zone-specific expression of these two enzymes can explain zone-dependent steroidogenesis. In animals with only one CYP11B, other enzymes must have a zone-dependent expression to maintain zone-specific steroidogenesis.[8,11,12,15] In humans (Fig 1), 17α-hydroxylase/17,20-lyase (CYP17A1) is known to play an important role in functional zonation: it is absent in the zG which facilitates aldosterone production, it is present in the zF in which it predominantly executes its 17-hydroxylase activity necessary for cortisol production, and it is also present in the zR in which it is a cofactor for adrenal androgen production through its 17,20-lyase reaction.[16] Adult rats, mice, rabbits and hamsters do not express CYP17A1 and therefore corticosterone, and not cortisol, is the end-product in their glucocorticoid pathway.[3,17,18]

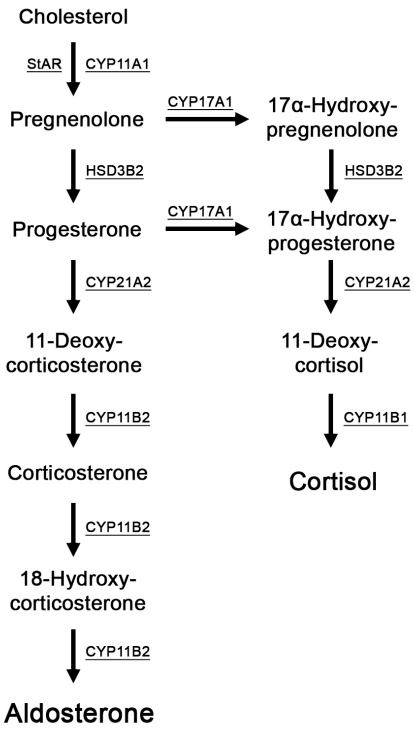

Figure 1 Human adrenocortical steroidogenesis in the zona glomerulosa and zona fasciculata. StAR, steroidogenic acute regulatory protein; CYP11A1, cytochrome P450 side chain cleavage; HSD3B2, 3β-hydroxysteroid dehydrogenase B2; CYP17A1, 17α-hydroxylase/17,20-lyase; CYP21A2, 21-hydroxylase; CYP11B1, 11β-hydroxylase cytochrome P450; CYP11B2, aldosterone synthase. Adapted from Payne and Hales[6] and Pihlajoki et al.[37]

The aim of this study was to study the number of *CYP11B* genes in the canine genome, and to determine which (other) enzymes are zone-specific in the canine adrenal cortex. We therefore studied (1) gene expression in isolated zF and zG tissue to determine the relative mRNA expression of steroidogenic enzymes and their major regulators: the adrenocorticotropic hormone receptor (*MC2R*) and Steroidogenic factor 1 (*SF-1*), and (2) the large sequence gap near the so-called *CYP11B2* gene on chromosome 13 to determine whether dogs have one or multiple *CYP11B* genes.

Materials and methods

Animals and tissues

For this study, normal adrenal glands were used, obtained from 10 healthy dogs. The The dogs were euthanized for reasons unrelated to the present study, approved by the Ethical Committee of Utrecht University. All dogs were of mixed breed from two to six years of age, all dogs were intact, six dogs were female and four dogs were male. For immunohistochemistry (IHC), five adrenal glands were fixed in 4% buffered formalin. After 24 to 48 hours of fixation, the tissues were embedded in paraffin and cut into 5-µm sections, after which they were mounted on SuperFrost Plus microscope slides. For microdissection, fresh tissue from five adrenal glands was snap frozen in liquid nitrogen within 10 min after resection and stored at -70°C until further use.

Microdissection

Cryosections of 8 µm were made and five to eight sections were mounted on an MMI MembraneSlide. The slides were directly processed or stored at -70°C, for a week at maximum. To ensure that enough RNA was left in the frozen sections, one section was directly put in RLT plus buffer (QIAGEN) and stored in a -20°C freezer until RNA isolation. The frozen sections from the -70°C freezer were defrosted at room temperature, quickly fixed in 70% isopropyl alcohol and stained with 1:1 haematoxylin for correct orientation of the tissue. The sections were quickly washed with milliQ and dehydrated with 70% isopropyl alcohol, 100% isopropyl alcohol and xylene. Areas of zG and zF were cut with Laser Microdissection (MMI CellCut) with a Nikon Eclipse TE300 microscope, 10^6 µm^2 per zone. To ensure that exclusively the desired zone was cut, areas from the zG were cut from the center of the zG and areas from the zF were cut from the center of the upper half of the zF. The zones were collected in tubes with sticky caps (MMI) and 60 µl RLT plus buffer (QIAGEN) was added to the tissue after which it was stored at -20°C. RNA isolation was carried out with the QIAGEN RNeasy Micro Kit according to protocol. RNA quality was analyzed with the BioAnalyzer Pico Total RNA Kit. NuGEN Ovation PicoSL WTA System V2 was used to amplify RNA. SPIA (Single Primer Isothermal Amplification, NuGEN) cDNA was stored at -20°C. The SPIA cDNA concentration was measured with NanoDrop.

Primer design

All primers for qPCR were based on mRNA sequences of our genes of interest that were obtained from the NCBI GenBank database. Primers (Table 1) were designed using Perl-primer (v1.1.14: Copyright 2003-2006 Owen Marshall, http://perlprimer.sourceforge.net/) software according to the parameters in the Bio-Rad (Veenendaal, The Neth-

Table 1 Primer pairs

Target gene		Sequence (5' > 3')	T[a] (°C)	Accession number	Length product
AGTR2	For	GGT GCT ATT ACG TCC CCG AG	56.8	XM_549206.3	205
	Rev	CTG ATG GTG GCA AGG GTG AT			
DAB2	For	TGC AAC CCT ACA GAC CAA CC	62.0	XM_536493.4	179
	Rev	GAC CAC TCA TCA TGG CTC CC			
Wnt4	For	CGA GGA GTG CCA GTA CCA GT	61.0	XM_005617834.1	124
	Rev	AGA GAT GGC GTA CAC GAA GG			
PR	For	CAA TGG AAG GGC AGC ATA AC	58.5	NM_001003074.1	103
	Rev	CAG CAC TTT CTA AGG CGA CA			
MC2R	For	TCA TGT GGT TTT GCC GGA AGA GAT	58.5	XM_003638756.1	138
	Rev	AAT GGC CAG GCT GCA AAT GAA A			
SF-1	For	AGG GCT GCA AGG GGT TTT TCA A	59.0	XM_846937.2	143
	Rev	CAT CCC CAC TGT CAG GCA CTT CT			
StAR	For	CTC TGC TTG GTT CTC GG	62.5	NM_001097542.1	125
	Rev	CCT TCT TCC AGC CTT CC			
CYP11A1	For	CAC CGC CTC CTT AAA AAG TAA CAA G	63.3	XM_535539.3	129
	Rev	GCT GCG TGC CAT CTC GTA G			
HSD3B2	For	CAG GAG GGT TTC TGG GTC AG	56.5	NM_001010954	186
	Rev	AGG CTC TCT TCA GGC ACT GC			
CYP17A1	For	CCT GCG GCC CCT ATG CTC	60.0	XM_535000.3	134
	Rev	GGC CGG TAC CAC TCC TTC TCA			
CYP21A2	For	AGC CCG ACC TTC CCC TCC ACC TG	64.5	NM_001003335	152
	Rev	TCT GCC GGC GAA GTC CAC CCA TTT			
CYP11B2	For	GCC TAC CCC TTG TGG ATG AC	62.0	XM_00343180587.1	126
	Rev	CTC TGT GAC TGC TGT CTG GG			
RPS5	For	TCA CTG GTG AGA ACC CCC T	62.5	XM_533568	141
	Rev	CCT GAT TCA CAC GGC GTA G			
RPS19	For	CCT TCC TCA AAA AGT CTG GG	61.0	XM_005616513	95
	Rev	GTT CTC ATC GTA GGG AGC AAG			
SRPR	For	GCT TCA GGA TCT GGA CTG C	61.2	XM_546411	81
	Rev	GTT CCC TTG GTA GCA CTG G			
SDHA	For	GCC TTG GAT CTC TTG ATG GA	61.0	DQ402985	92
	Rev	TTC TTG GCT CTT ATG CGA TG			
Probe *CYP11B2*	For	GTC GCT ACA GGC CGT CCA GAA GT	63.7	XM_00343180587.1	307
	Rev	GCC CAG CCG CTC CCC GAA CAG			

Primer pairs for RT-qPCR analysis of *AGTR2, DAB2, Wnt4, PR, MC2R, SF-1, StAR, CYP11A1, HSD3B2, CYP17A1, CYP21A2,* the so-called *CYP11B2*, and the reference genes *RPS5, RPS19, SRPR*, and *SDHA*, and a primer pair designed to create a probe for *CYP11B1/2*, positioned halfway into exon 3 through halfway into exon 4. All positions are based on canine mRNA sequences, as published in the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/). Length product indicated in number of base pairs. For, forward primer; Rev, reverse primer; T[a], annealing temperature.

erlands) iCycler manual, checked for secondary structure formation with mfold web server v3.1, and ordered from Eurogentec (Maastricht, The Netherlands). Optimization and confirmation of primer specificity of the PCR reaction was performed as described previously.[19]

RT-qPCR

To determine and compare the expression levels of zG markers angiotensin II receptor 2 (*AGTR2*), Disabled-2 (*DAB2*), Wingless-type MMTV integration site family, member 4 (*Wnt4*), and progesterone receptor (*PR*), of steroidogenic factors *MC2R* and *SF-1*, and of steroidogenic enzymes steroidogenic acute regulatory protein (*StAR*), cytochrome P450 side chain cleavage (*CYP11A1*), 3β-hydroxysteroid dehydrogenase B2 (*HSD3B2*), *CYP17A1*, 21-hydroxylase (*CYP21A2*), and the so-called *CYP11B2* between the zG and the zF, RT-qPCR analyses were performed on 0.25 µg/µL amplified SPIA cDNA. Ribosomal protein S5 (*RPS5*), ribosomal protein S19 (*RPS19*), small proline-rich protein (*SPRP*), and succinate dehydrogenase complex subunit A (*SDHA*) were used as reference genes to correct for differences in cDNA concentration.[20–22] SYBRgreen supermix (Bio-Rad) was used for detection, and CFX Manager 3.0 (Bio-Rad) was used to analyze the data. GeNorm software (https://genorm.cmgg.be/) was used to analyze relative expression levels of the reference genes, which justified their use. The $2^{-\Delta\Delta Ct}$ method[23] was used to calculate the normalized relative expression of each target gene.

Southern blot

The Bacterial Artificial Chromosome (BAC) clones used were CH2-265M08 (M08) and CH82-485G10 (G10), as ordered from the BACPAC Resources Center, Pieter de Jong's Laboratory, of CHORI-82. On the G10 BAC, the so-called *CYP11B2* is present. The M08 BAC contains both the so-called *CYP11B2* gene and the sequence gap. Both BACs were cultured on agar plates. Of the cultured bacteria, a colony pick was inoculated into LB-Miller medium with 1% chloramphenicol and kept at 37°C with agitation overnight. DNA was isolated with QIAGEN Maxi Kit, according to protocol. Both BACs were cut with restriction enzyme BamHI (Promega, Leiden, The Netherlands); the restriction site for BamHI is 5' G↓GATCC 3' - 3' CCTAG↑G 5'. Approximately 500 ng per BAC was used and electrophoresed on a 0.8% agarose TAE gel overnight. Southern blot was performed according to protocol of Davis et al.[24] A probe was made with the primers shown in Table 1, which were designed using PrimerSelect of DNASTAR Lasergene 12, and ordered from Eurogentec. SYBRgreen supermix (Bio-Rad) was used for amplification, and a temperature gradient was performed to determine the optimal annealing temperature. A C1000 Touch thermal cycler (BioRad) was used for all PCR reactions. The product was put on a 1% agarose TBE gel and checked for correct product size after electrophoresis, after which the DNA was isolated from the gel with QIAquick Gel Extraction Kit (QIAGEN) according to the protocol. The *CYP11B* probe was labeled with the Thermo Scientific Biotin DecaLabel DNA Labeling Kit according to the protocol. With the North2South Chemiluminescent Hybridization and Detection Kit, the southern blot membrane was hybridized

overnight with the labeled probe of which attachment was subsequently detected with the same kit, according to the protocol.

Analysis sequence gap

In the canine genome as documented in the NCBI GenBank database, near to the so-called *CYP11B2* gene, a region of approximately 50,000 bp (Chr13: 39,840,000–39,890,000) is presented as a sequence gap, which is present on BAC CH82-265M08 (M08). Of this M08 BAC, a draft sequence containing 36 unordered contigs, an unordered set of partly overlapping DNA segments, can be found under accession number AC183568.1. Using MegAlign from DNASTAR Lasergene 12, the contigs were aligned with the known M08 sequence, which left contigs corresponding to the sequence gap. Using the NCBI BLAST tool (blast.ncbi.nlm.nih.gov), these contigs were blasted against the canine so-called CYP11B2 gene. For analysis of the amino acid sequence of the known canine CYP11B enzyme, accession number XP_539192.3 was used.

Immunohistochemistry

The paraffin-embedded sections were rehydrated in a series of xylene and ethanol baths. Antigen retrieval was achieved by using a citrate buffer (0.01 M, pH 6.0, 0.05% tween) for CYP17A1 or Tris/EDTA (10 mM Tris base, 1 mM EDTA, 0.05% tween, pH 9.0) for Wnt4 at 98°C for 10 minutes. The slides were immersed in 3.5% (CYP17A1) or 0.35% (Wnt4) H2O2 in Tris buffered saline with 0.1% tween (TBST0.1%, 0.02 M, pH 7.6) for 30 minutes to block endogenous peroxidase; 5–10% normal goat serum (NGS) in 1% bovine serum albumin (BSA) was applied to the slides to block nonspecific binding sites. The slides were incubated with the anti-CYP17A1 antibody (courtesy of A.J. Conley, rabbit-anti-bovine, 1 : 7,500) or the anti-Wnt4 antibody (rabbit-anti-human, 1 : 6,000) and kept at 4°C overnight. The following day, secondary anti-rabbit antibody (Envision, K4003) was applied for 50–90 minutes. Bound antibody was visualized with the DAB substrate kit for peroxidase (DAKO, K3468, Heverlee, Belgium), after which the slides were counterstained with hematoxylin, dehydrated in a series of ethanol and xylene baths, and embedded with Vecta-Mount Mounting Medium (H-5000).

Immunoprecipitation

For normal adrenal homogenate, protein was isolated from a normal canine adrenal gland using radioimmunoprecipitation (RIPA) buffer base. To isolate CYP17A1 protein out of normal adrenal homogenate, immunoprecipitation was performed with the anti-CYP17A1 antibody, with Dynabeads Protein A (Invitrogen, Life Science Technologies, Breda, The Netherlands). 250 µL of a normal adrenal gland homogenate (8.05 µg/µL) was incubated with 50 µL of 1 : 2,500 anti-CYP17A1 antibody; 20 mg of Protein A was dissolved in 200 lL phosphate-buffered saline and added to the homogenate/antibody solution. The solution was incubated for 30 minutes at room temperature and then centrifuged for 10 minutes at 1,000 rates per minute. The supernatant was removed and the sediment

was washed with TBST0.1% and again centrifuged. Fifty microliters of phosphate/citrate pH 3 was added to the remaining sediment and the concoction was incubated for 15 minutes at room temperature, and then centrifuged for 5 minutes at 13,400 rate per minute.

Western blot

To confirm specificity of the anti-CYP17A1 antibody, Western blot was performed using the immunoprecipitate from the previous paragraph (0.39 µg/µL), a normal adrenal homogenate (4 µg/µL), and a negative control containing only RIPA buffer base. All samples were diluted 1 : 1 with sample buffer with dithiothreitol and heated at 95°C for 2 minutes. A 10% acryl/bisacryl running gel was used; 20 µL of the diluted samples or 12 µL of the Precision plus Protein Standard (BioRad) was loaded onto the gel. After gel-electrophoreses, the gel was blotted onto a Hybond Enhanced Chemiluminescence (ECL) nitrocellulose membrane (Amersham, GE Healthcare, Diegem, Belgium). The membrane was blocked for 60 minutes in TBST0.1% with 4% ECL Blocking Agent, after which it was incubated overnight at 4°C in the anti-CYP17A1 antibody in a 1 : 2,500 concentration, diluted in TBST0.1% in 4% BSA. The next day, the membranes were incubated for

Table 2 RT-qPCR results

Gene	Mean FC zG	95% CI	Mean FC zF	95% CI	Ratio zG/zF	P-value
Zone-specific markers						
AGTR2	4.33	-0.07 to 8.72	0.29	-0.13 to 0.71	14.93	*0.014*
DAB2	2.02	0.69–3.34	0.51	0.33–0.69	3.96	*0.014*
Wnt4	7.11	0.09–14.12	0.73	-1.23 to 2.68	9.74	*0.05*
PR	3.98	2.69–5.28	1.15	-1.41 to 3.71	3.46	*0.027*
Steroidogenic factors						
MC2R	1.42	0.45–2.40	0.94	-0.36 to 2.25	1.51	0.142
SF-1	1.23	0.47–1.99	0.98	0.24–1.72	1.26	0.462
Steroidogenic enzymes						
StAR	0.77	0.36–1.18	1.61	1.05–2.16	0.48	*0.014*
CYP11A1	0.49	0.13–0.85	2.85	2.31–3.38	0.17	*0.014*
HSD3B2	0.92	0.25–1.59	1.42	0.50–2.34	0.65	0.221
CYP17A1	0.91	-0.85 to 2.67	4.26	0.14–8.38	0.21	*0.05*
CYP21A2	1.23	0.54–1.91	1.08	-0.10 to 2.25	1.14	0.624
CYP11B2	2.44	-0.37 to 5.25	1.72	0.13–3.31	1.42	0.624

RT-qPCR results for AGTR2, DAB2, Wnt4, PR, MC2R, SF-1, StAR, CYP11A1, HSD3B2, CYP17A1, CYP21A2, and the so-called CYP11B2. FC, fold change; zG, zona glomerulosa; zF, zona fasciculata; CI, confidence interval. P values ≤.05 were considered significant and are emphasized in italic.

60 minutes with the secondary antibody (anti-rabbit, horseradish peroxidase conjugated, 1 : 20,000). TBST0.1% was used for all washing steps. An ECL advanced Western blotting detection kit (Amersham RPN2135, GE Healthcare) was used for protein visualization, and chemiluminescence was detected using a ChemiDoc XRS Chemi Luminescent Image Capture (BioRad).

Statistical analyses

Statistical analyses were performed using SPSS22 (IBM, Armonk, NY). Due to the non-normal distribution of the variables, mRNA expression levels between groups were compared with the nonparametric Mann-Whitney U-test. Relative expression levels were compared between the zG and the zF, where the mean delta cycle threshold (CT) of all samples was used as reference point to calculate the $2^{-\Delta\Delta Ct}$.[23] A P-value ≤ 0.05 was considered significant.

Results

The relative mRNA expressions of *AGTR2* (P = 0.014), *DAB2* (P = 0.014), *Wnt4* (P = 0.05), and *PR* (P = 0.027) were significantly higher in the zG compared with the zF (Table 2), indicating successful separation of zG and zF tissue. Immunohistochemical staining confirmed the zG specificity of Wnt4 (Fig. 2).

RT-qPCR analysis revealed equal expression of the so-called *CYP11B2* gene in both zG and zF (Table 2), which could suggest that there is only 1 *CYP11B* gene in the dog. To

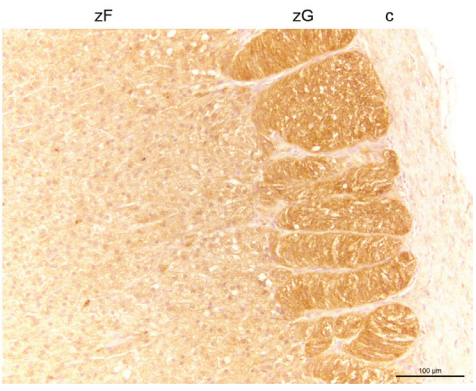

Figure 2 Immunohistochemical staining of Wnt4. Representative example of immunohistochemical staining for Wnt4 in a normal adrenal gland. Staining is most intense in the zG in a cytoplasmic granular pattern. Staining is faintly visible in the zF and capsule. Bar represents 100 µm. c, capsule; zG, zona glomerulosa; zF, zona fasciculata.

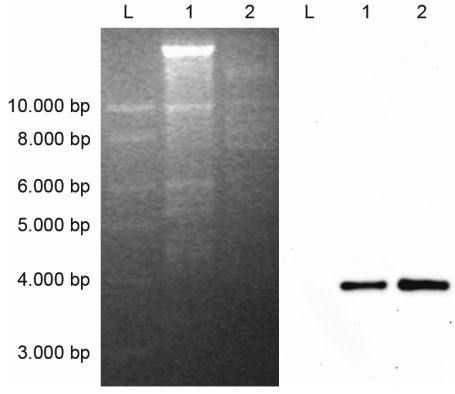

Figure 3 Southern blot. Left: EtBr staining of the gel. Right: After southern blot and hybridization. L: DNA ladder (Promega); lane 1: CH82-265M08 (M08) BAC; lane 2: CH82-485G10 (G10) BAC. Both BACs were cut with BamHI (Promega). Both BACs show only 1 signal at approximately 4,000 bp.

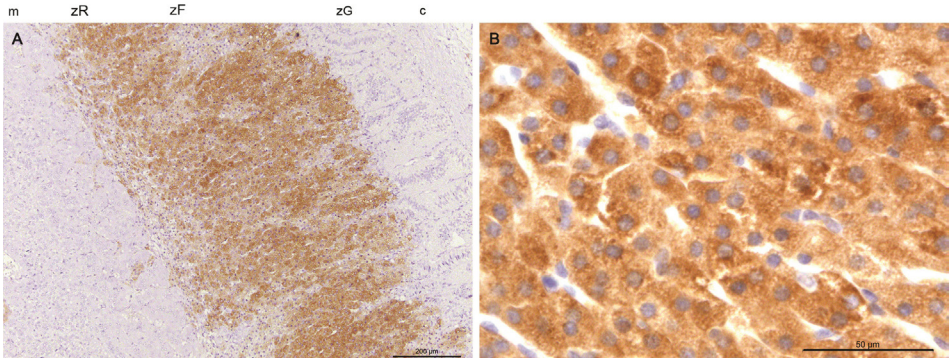

Figure 4 Immunohistochemical staining of CYP17A1. Representative examples of immunohistochemical staining for CYP17A1 in normal adrenal glands. (A) CYP17A1 positive cells detected throughout the entire zF and zR. Staining is absent in the capsule, zG, and medulla. Bar represents 200 µm. (B) Magnification with a 40x objective of the zF, showing a cytoplasmic granular staining pattern. Bar represents 50 µm. c = capsule, zG = zona glomerulosa, zF = zona fasciculata, zR = zona reticularis, m = medulla.

further prove this, we examined 2 BAC clones: 1 BAC containing the so-called *CYP11B2* gene, BAC G10; and 1 BAC containing the so-called *CYP11B2* gene plus the sequence gap, BAC M08. BamHI was used as restriction enzyme to cut the BACs. The predicted fragment size containing the so-called *CYP11B2* gene was 3,900 bp, based on the BAC's sequence information. Southern blot analysis of the BACs revealed a single restriction fragment which corresponded to the predicted fragment size that hybridized to the *CYP11B* probe in both BACs (Fig. 3).

Two predicted protein isoforms can be found in the NCBI database corresponding to the canine so-called *CYP11B2* gene, but the names change to CYP11B1 on protein level. The amino acid sequences of these proteins do correspond to the so-called *CYP11B2* gene. Analysis of the amino acid sequences showed a proline at residue 112, a glycine at residue 288, and an alanine at residue 320.

Next, we analyzed the 36 unordered contigs from the BAC M08 to determine whether (parts of) an extra *CYP11B* gene was present in the sequence gap. Of the 36 unordered contigs, 14 contigs could be aligned along the sequenced parts of M08. This left the other contigs to belong to the sequence gap. When blasting the exons of the so-called *CYP11B2* gene against these gap contigs, exon 1 was found full length on contigs 11, 32, and 34 (97–99% similarity), approximately half of exon 2 (first 85 out of 156 bp) was found on the same contigs (96% similarity in all 3 contigs), a large part of exon 9 (1,262–1,264 out of 1,589 bp) was found on contigs 32 and 34 (92 and 91% similarity), and a small part of exon 9 (158 out of 1,589 bp) was found on contig 11 (86% similarity). On these 3 contigs, exon 2 was quickly followed by exon 9. Exons 3–8 were not found on any of the contigs belonging to the sequence gap.

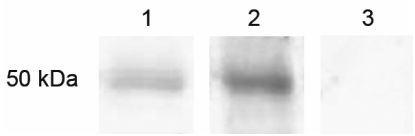

Figure 5 Western blot CYP17A1. Lane 1: immunoprecipitate. Lane 2: normal adrenal homogenate. Lane 3: negative control. At approximately 50 kDa, CYP17A1 bands are visible in lane 1 and lane 2, but not in the negative control. kDa, kilodalton; CYP17A1, 17α-hydroxylase/17,20-lyase.

The relative mRNA expressions of *StAR* (P = .014), *CYP11A1* (P = .014), and *CYP17A1* (P = .05) were significantly higher in the zF compared with the zG (Table 2). Immunohistochemical staining for CYP17A1 demonstrated clear cytoplasmic granular staining in the zF and the zR in all sections (Fig. 4). In the capsule, the zG, and the medulla, the staining was absent. In both the normal adrenal homogenate and the immunoprecipitate, Western blotting for CYP17A1 revealed a band at approximately 50 kDa (Fig. 5), corresponding to CYP17A1's expected weight.[25] The normal adrenal homogenate produced a more intense signal compared with the immunoprecipitate, which can be explained by the higher protein concentration in the normal adrenal homogenate. No band was visible in the negative control. The relative mRNA expression of *HSD3B2, CYP21A2, MC2R*, and *SF-1* did not differ significantly between the two zones (Table 2).

Discussion

This study is the first to demonstrate the zone-specific expression of steroidogenic enzymes in the canine adrenal cortex. Studying the expressions of CYP11B1 and CYP11B2 in the canine adrenal cortex was challenging, as the sequence of only 1 gene ("*CYP11B2*") is known. Our RT-qPCR results of the so-called *CYP11B2* showed equal expression in the zG and zF, suggesting that the canine adrenal cortex expresses only 1 *CYP11B* gene. The significantly higher expression of *AGTR2*,[26] *DAB2*,[27] *Wnt4*,[28] and *PR*[29,30] in the zG compared with the zF justified their use as canine zG markers and confirmed correct isolation of the respective zones. Equal *CYP11B2* distribution could thus not be attributed to incorrect zone isolation.

In the canine genome, near the known sequence of the so-called *CYP11B2* gene, a large sequence gap is present. Because the *CYP11B* gene duplicates in humans and mice lie very close to each other,[6] we questioned whether this sequence gap could be the place for another *CYP11B* gene copy. Southern blot results with a probe from exons 3 to 4 rejected this, as no complete *CYP11B* gene copy was present in the sequence gap on the M08 BAC. Hypothetically, an existent *CYP11B* gene copy could be mutated in a way that it still codes for a functional *CYP11B* enzyme, but it was not similar enough to the known *CYP11B* gene in our Southern blot probe to recognize it. Another option is that there is another *CYP11B* gene copy which is not located on the M08 BAC, but somewhere else in a nonsequenced part of the canine genome. However, this seems unlikely, since the gene duplicates in humans and mice are separated by no more than 40 kb and 8 kb, respectively.[6] A gene knock-out study (siRNA of the so-called *CYP11B2*) to analyze aldosterone and cor-

tisol concentrations would likely produce a more definitive conclusion as to whether there is only 1 *CYP11B* gene in the dog.

Curnow et al.[31] state that the amino acid sequence of a CYP11B protein can predict whether the enzyme has an aldosterone synthase activity. They found that a CYP11B1 enzyme which has a serine to glycine substitution at residue 288 (Ser288Gly) and a Val320Ala is a fully functioning aldosterone synthase.[7,31] An Ile112Pro in human CYP11B2 has been shown to give a 3-fold increase of 11β-hydroxylation activity and a 2-fold increase of 18-hydroxylation.[7] Analysis of the known canine CYP11B amino acid sequence showed the presence of both a glycine at residue 288 and an alanine at residue 320, thereby suggesting that the corresponding enzyme has aldosterone synthase activity. Furthermore, the presence of a proline at residue 112 in the known canine CYP11B suggests that this enzyme has a higher 11β-hydroxylase activity than the human CYP11B2, which is convenient for cortisol production. The combined findings imply that the known canine CYP11B concerns an enzyme which has aldosterone synthase activity but is also efficient in cortisol production.

Interestingly, our search for analogous exons of *CYP11B* in the sequence gap of the unordered contigs showed fragments of exons 1, 2, and 9, but not exons 3–8. The exon 2 fragment was closely or directly followed by the exon 9 fragment. Pseudogenes have been reported in, among others, guinea pigs,[32] rats,[33] and cows.[34] Rats have 4 *CYP11B* genes in total: *CYP11B1, CYP11B2, CYP11B3*, and *CYP11B4*, of which *CYP11B3* is very similar to *CYP11B1*, and *CYP11B4* is a pseudogene which misses exon 3 and part of exon 4. Cows have no less than 5 *CYP11B* genes,[34] of which 2 have similar enzymatic activities (both are called *CYP11B1*) and 3 are pseudogenes. The presence of exons 1, 2, and 9 in the sequence gap most likely indicates remnants of a canine *CYP11B* pseudogene.

Birds and amphibians possess only 1 *CYP11B* gene: *CYP11B1*.[11,12] This *CYP11B1* seems to be a common ancestral gene of which duplication and specialization is restricted to mammals and has occurred in at least primates and rodents.[11,32] This specialization has resulted in a partial loss of function of one of the gene duplicates, since the ancestral CYP11B1 enzyme was able to catalyze 11β-hydroxylation, 18-hydroxylation, and 18-oxidation, while the primate and rodent CYP11B1 can only perform 11β-hydroxylation. Primate and rodent CYP11B2 is more similar to the ancestral enzyme than CYP11B1, as CYP11B2 can catalyze all 3 reactions (Fig. 1).[6] The presence of multiple functional CYP11B enzymes and *CYP11B* pseudogenes in different species could indicate that duplication of the *CYP11B* gene occurred in a common ancestor mammal, and that loss of function, silencing, or partial deletion of 1 or multiple *CYP11B* genes occurred independently in the following evolution of the different species. Hypothetically, this might have occurred in canine spp. as well. The results of this study suggest that there is only 1 *CYP11B* gene present in the canine genome. Zone-dependent steroid synthesis must therefore depend on a different zone-specific enzyme.

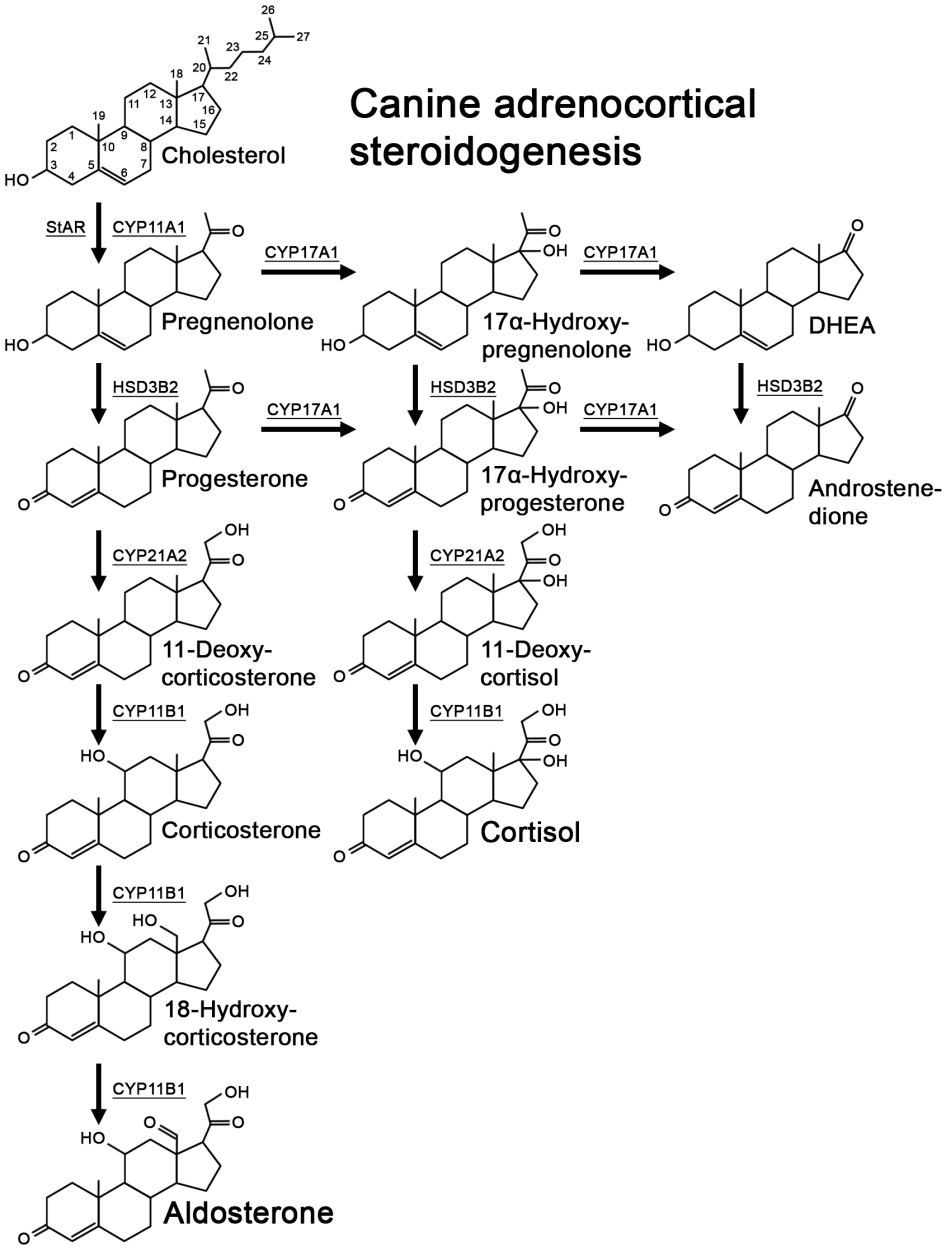

Figure 6 Proposed canine adrenocortical steroidogenesis. StAR, steroidogenic acute regulatory protein; CYP11A1, cytochrome P450 side chain cleavage; HSD3B2, 3β-hydroxysteroid dehydrogenase 2; CYP17, 17α-hydroxylase/17,20-lyase; CYP21A2, 21-hydroxylase; CYP11B1, 11β-hydroxylase cytochrome P450.

Relative mRNA *CYP17A1* expression was significantly higher in the zF compared with the zG, which was confirmed by positive CYP17A1 IHC staining in zF and zR only. This is similar to human and guinea pig adrenals, in which CYP17A1 is expressed in the zF and zR, but not the zG.[35,36] Zone-specific expression of CYP17A1 is an important factor in canine zone-specific steroidogenesis, providing an explanation for the fact that cortisol synthesis is limited to the zF and zR. While rats and mice are widely used as models for human steroidogenesis, these animals do not express CYP17A1 in the adrenal cortex and therefore cannot produce cortisol. Ferrets do express CYP17A1, but their zonation pattern is different from that of humans.[37] The fact that canine adrenal zonation is similar to that of humans and dogs also express CYP17A1, and therefore have the same end product of the glucocorticoid pathway, indicates that dogs provide a better model for human steroidogenesis than rodents.

Since CYP11A1 and StAR are necessary for the first steps in steroidogenesis, i.e., for both aldosterone and cortisol production, the significantly higher relative expression of *StAR* and *CYP11A1* in the zF compared with the zG was not expected. However, although multiple studies in rats and humans have shown no difference in *StAR* expression between the zG and zF,[38–40] Peters et al.[41,42] describe that in nonstimulated rats (i.e., not stimulated to produce extra aldosterone with a high potassium diet), *StAR* mRNA expression is most prominent in the zF and zR, whereas the zG shows less *StAR* expression. Dogs used in this study received a regular diet with normal potassium content and were thus not stimulated to produce extra aldosterone. Higher expression of StAR in the zF than in the zG could be explained by the fact that the transport of cholesterol to the inner mitochondrial membrane, as facilitated by StAR, is the rate-limiting step in steroidogenesis.[43–45] CYP11A1 has also been documented to play a rate-limiting role in steroidogenesis.[5,6] Higher expression of CYP11A1 and StAR in the zF than in the zG could ensure the greater production rates of glucocorticoids compared with mineralocorticoids in dogs.

Interestingly, bovine CYP11A1 has been documented to modulate CYP11B1 activities, while human CYP11A1 does not affect the product pattern of bovine or human CYP11B1 or -2. Cao and Bernhardt (1999) found that cotransfection of bovine CYP11A1 with CYP11B1 resulted in an increase of 11β-hydroxylation and a decrease of 18-hydroxylation and 18-oxidation.[46,47] This could be due to a direct interaction of CYP11A1 with CYP11B1, which causes conformational changes in CYP11B1 that result in a different binding of the substrates and intermediates.[7,46] Possibly, in species which possess only 1 CYP11B enzyme, higher CYP11A1 expression in the zF stimulates 11β-hydroxylation and inhibits 18-hydroxylation and 18-oxidation, while this inhibition is absent in the zG due to lower CYP11A1 expression.

CYP21A2 and HSD3B2 are essential for both glucocorticoid and mineralocorticoid synthesis and have no ratelimiting functions.[1,48] The results of this study demonstrated no difference in their expression between the zones, in agreement with the known literature. ACTH is known to be responsible for glucocorticoid production in the zF,[49] but an acute

rise in circulating ACTH concentration is also able to stimulate aldosterone secretion. This requires the presence of MC2R in the zG,[50,51] in concordance with the results of this study.

In humans, mice, and rats, SF-1 is known to be localized in all 3 layers of the adrenal cortex.[52] In humans, SF-1 is essential for full expression of, among others, MC2R, StAR, CYP11A1, HSD3B2, CYP17A1, CYP21A2, and CYP11B1, but not CYP11B2. The lack of CYP11B2 stimulation by SF-1 is most likely due to a different transcription mechanism.[53] A recent study from our group found immunohistochemical SF-1 staining throughout the entire normal canine adrenal cortex.[54] As expected, this study showed no difference in SF-1 expression between the zF and zG in canine adrenals.

In conclusion, in this first study concerning canine steroidogenesis, we propose a canine steroidogenesis scheme (Fig. 6) with only 1 CYP11B enzyme, which implies that the zone-specific production of aldosterone and cortisol is probably due to zone-specific CYP17A1 expression. CYP17A1 expression in the zF is crucial for cortisol synthesis, while lack of CYP17A1 in the zG restricts steroidogenesis to mineralocorticoid production. The zone-specific presence of CYP17A1 makes it an attractive target for selective inhibition of cortisol synthesis without affecting mineralocorticoid production in the zG.

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Conflict of Interest Declaration: Authors declare no conflict of interest.
Off-label Antimicrobial Declaration: Authors declare no off-label use of antimicrobials.

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Part

II

New Treatment Options: *In Vitro* Studies

Chapter

4

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

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Abstract

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's 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 ± 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.

Introduction

Cushing's 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's 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 (mifepris-

tone). 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.

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 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% CO2 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 radioimmunoassay (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 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.

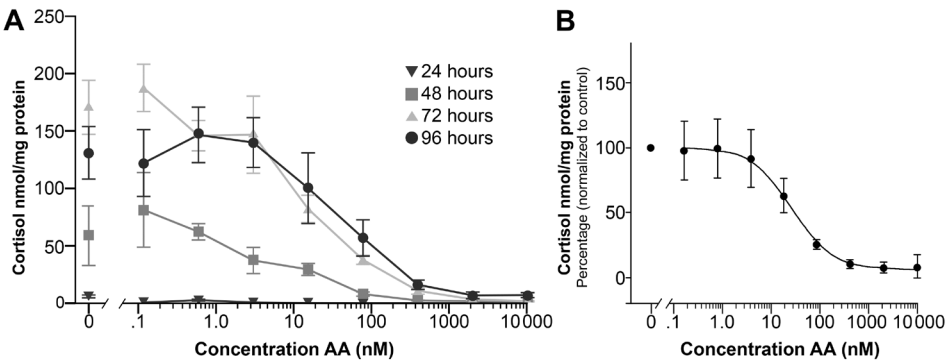

Figure 1 Determination of the optimal effects of AA on cortisol production as measured with radioimmunoassay. (A) To determine the optimal incubation time, canine primary adrenocortical cells (primary cell culture, n = 1) were incubated in quadruplicate with increasing concentrations of AA (0.125 nM 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.

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.

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).

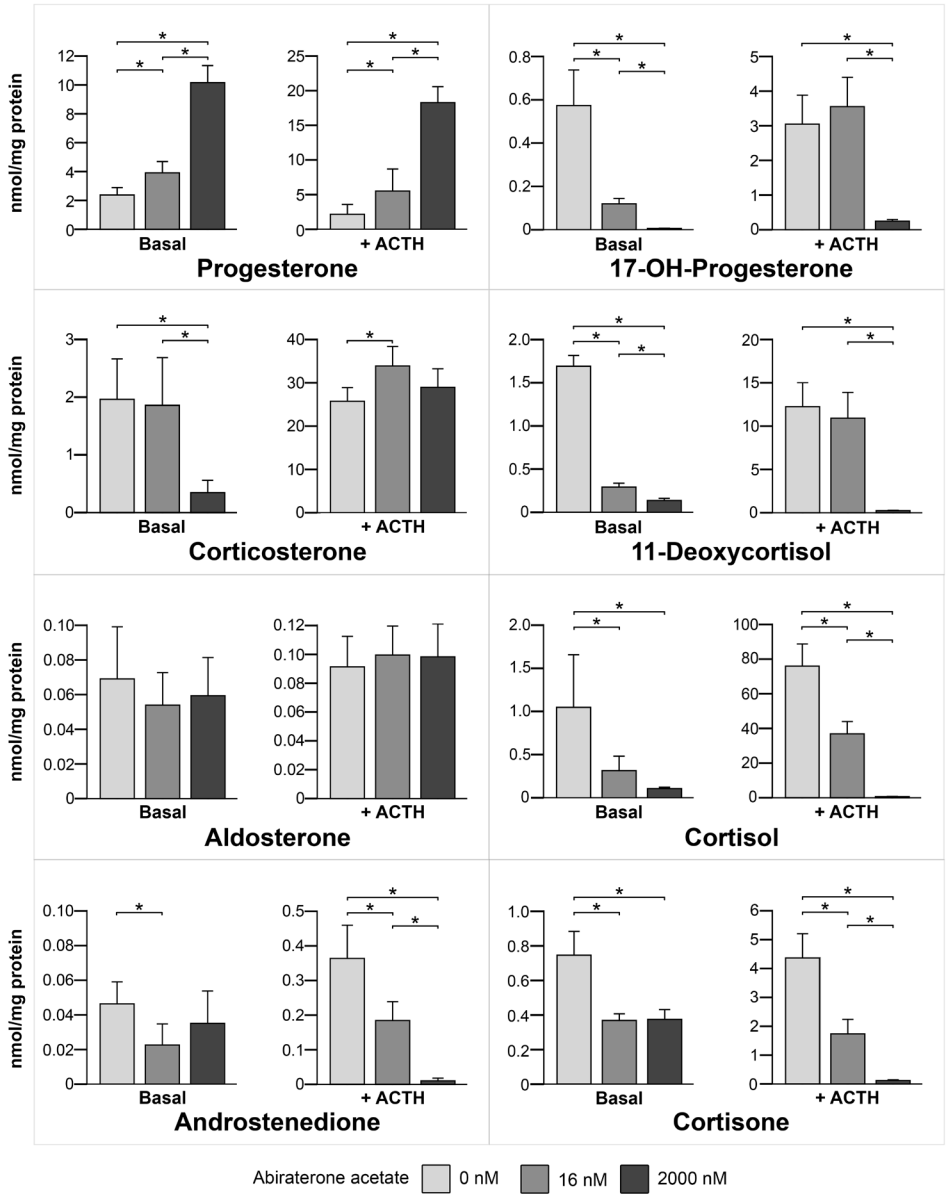

Figure 2 The effects of AA on steroid hormone production as measured with LC-MS/MS. Steroid hormone production was measured in canine primary adrenocortical cell cultures (n = 9) under basal and ACTH-stimulated conditions. Data are shown as mean ± SEM of nanomole of hormone per milligram protein. Scale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

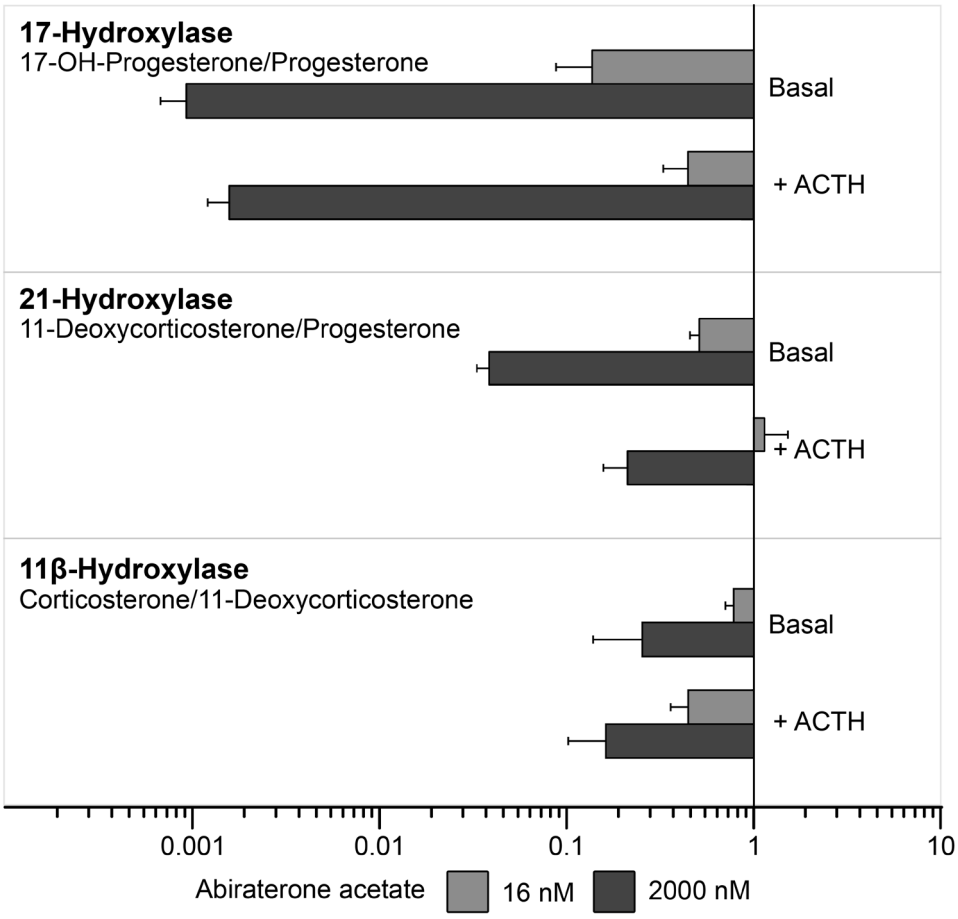

Figure 3 The effects of AA on steroid hormone ratios. Steroid hormone production was measured with LC-MS/MS in canine primary adrenocortical cell cultures (n = 4) under basal and ACTH-stimulated conditions. Ratios were calculated between the indicated hormones. The results were calculated in fold change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

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 ± 5.5 nmol/mg protein; 2000 nM AA, 4.8 ± 0.9 nmol/mg protein), whereas under ACTH-stimulated conditions AA increased the mean 11-deoxycorticosterone concentration (0 nM AA, 16.8 ± 10.9 nmol/mg protein; 2000 nM AA, 33.5 ± 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.

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)

Target 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). CT, cycle threshold; *StAR*, steroidogenic acute regulatory protein; *CYP11A1,* cytochrome P450 side chain cleavage; *CYP17A1*, 17α-hydroxylase/17,20-lyase; *HSD3B2*, 3β-hydroxysteroid hydrogenase type 2; *CYP21A2*, 21-hydroxylase; *CYP11B1*, 11β-hydroxylase.

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 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 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.

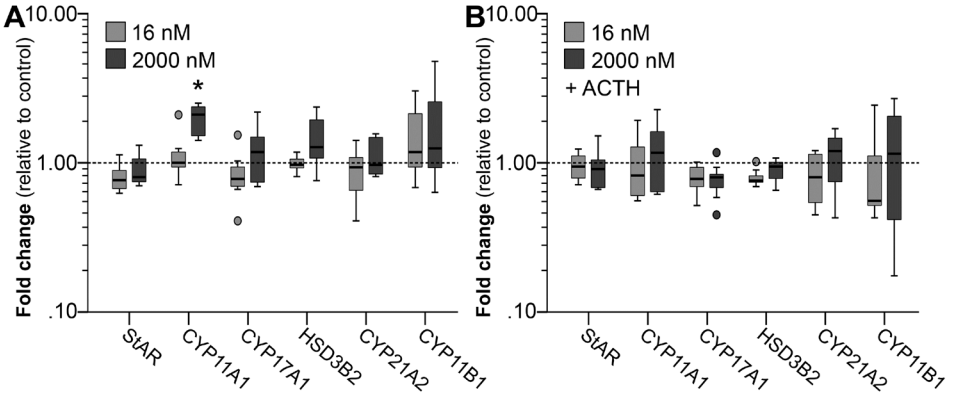

Figure 4 The effects of AA on the mRNA expression of steroidogenic enzymes. Gene expression was measured in canine primary adrenocortical cell cultures (n = 9) under (A) basal and (B) ACTH-stimulated conditions. Data are shown as mean ± SEM fold change relative to the corresponding DMSO vehicle control. The dotted line represents the control. The circles above and below the box plots indicate outliers. *P < 0.05. *StAR*, steroidogenic acute regulatory protein; *CYP11A1,* cytochrome P450 side chain cleavage; *CYP17A1*, 17α-hydroxylase/17,20-lyase; *HSD3B2*, 3β-hydroxysteroid hydrogenase type 2; *CYP21A2*, 21-hydroxylase; *CYP11B1*, 11β-hydroxylase.

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 inhibited, its capacity remains extensive.

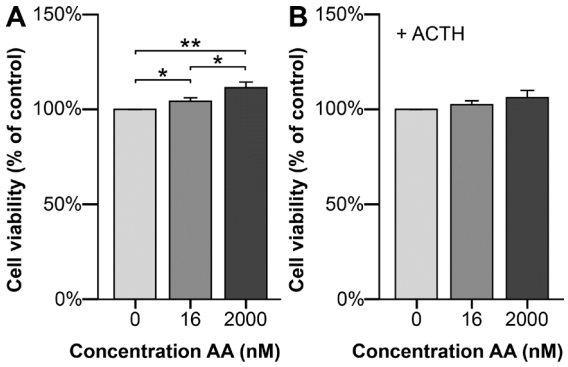

Figure 5 The effects of AA on cell viability. Cell viability was measured with alamarBlue™ assay in canine primary adrenocortical cell cultures (n = 9) under (A) basal and (B) ACTH-stimulated conditions. Data are shown as mean ± SEM of the percentage of detected fluorescence with each DMSO vehicle control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

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 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-deoxycorticosterone), 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]

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.

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Chapter

5

Melanocortin 2 Receptor Antagonists in Canine Pituitary-Dependent Hypercortisolism: *In Vitro* Studies

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Abstract

Canine hypercortisolism is most often caused by an ACTH-secreting pituitary adenoma (pituitary-dependent hypercortisolism; PDH). An interesting target for a selective medical treatment of PDH would be the receptor for ACTH: the melanocortin 2 receptor (MC2R). In this study we investigated whether two peptide compounds, BIM-22776 (#776) and BIM-22A299 (#299), are effective MC2R antagonists *in vitro*. Their effects on cortisol production and mRNA expression of steroidogenic enzymes, *MC2R* and melanocortin 2 receptor accessory protein (*MRAP*) were evaluated in primary adrenocortical cell cultures (n = 8) of normal canine adrenal glands. Cortisol production stimulated by 50 nM ACTH was dose-dependently inhibited by #299 (inhibition 90.7 ± 2.3% at 5 μM) and by #776 (inhibition 38.0 ± 5.2% at 5 μM). The ACTH-stimulated mRNA expression of steroidogenic enzymes, *MC2R* and *MRAP* was significantly inhibited by both compounds, but most potently by #299. These results indicate that canine primary cell culture is a valuable *in vitro* system to test MC2R antagonists, and that these compounds, but especially #299, are effective MC2R antagonists *in vitro*. To determine its efficacy *in vivo*, further studies are warranted. Antagonism of the MC2R is a promising potential treatment approach in canine PDH.

Introduction

Hypercortisolism (Cushing's syndrome) is one of the most frequently diagnosed endocrinopathies in dogs.[1] This serious endocrine disorder is characterized by chronic exposure to excessive amounts of glucocorticoids, which can be caused by either glucocorticoid administration or endogenous cortisol overproduction. Endogenous hypercortisolism occurs in approximately 1 to 2 per 1000 dogs per year,[2,3] and is most frequently (~80-85%) caused by an ACTH-producing pituitary adenoma (pituitary-dependent hypercortisolism; PDH).[1]

The current drug of choice for the medical treatment of canine PDH is trilostane, which competitively inhibits the steroidogenic enzyme 3β-hydroxysteroid dehydrogenase type 2 (HSD3B2).[4,5] However, HSD3B2 is required for all classes of adrenocortical hormones, and trilostane therefore does not only inhibit the production of cortisol but also that of

aldosterone.[6,7] Although trilostane is generally well tolerated, hypoadrenocorticism can occur,[8] and adrenal necrosis might occur more commonly than generally thought,[9] possibly due to increased ACTH secretion.[10] A more selective treatment option where the negative effects of increased ACTH secretion are countered could therefore improve the current medical treatment of canine PDH.

An interesting target for a more selective medical treatment of PDH would be the receptor for ACTH: the $G_{s\alpha}$-protein-coupled melanocortin 2 receptor (MC2R).[11] The MC2R is expressed in all zones of the adrenal cortex, but its major function is to stimulate the zona fasciculata cells to produce cortisol.[12,13] The MC2R is one of five melanocortin receptors: MC1R-MC5R, which are all activated by melanocortin peptides that are derived from the precursor pro-opiomelanocortin.[14] The MC2R is unique in its ligand selectivity: while multiple melanocortin peptides can bind to the other MC receptors, only ACTH binds to the MC2R.[15,16] The MC2R needs to be transported from the endoplasmic reticulum to the cell surface, for which it requires the melanocortin 2 receptor accessory protein (MRAP). MRAP forms a complex with the MC2R which allows the MC2R to leave the endoplasmic reticulum and reach the cell surface, and which is necessary for binding of ACTH to the MC2R.[15,17] This binding activates the cAMP-protein kinase A pathway which facilitates cholesterol transport to the inner mitochondrial membrane and the phosphorylation of several transcription factors. These activated transcription factors then increase the availability of free cholesterol and the transcription of genes encoding for steroidogenic enzymes, which eventually results in increased cortisol production.[18,19]

Consequently, a potent MC2R antagonist would be a great new treatment option to selectively inhibit ACTH-dependent hypercortisolism. The aim of this study was to evaluate whether the two peptide compounds BIM-22776 (#776) and BIM-22A299 (#299) are potent MC2R antagonists *in vitro*, and to determine whether MC2R antagonists have potential as a future treatment option for canine PDH.

Materials & Methods

Animals & Tissues

The adrenal glands of eight healthy dogs were used. The dogs were euthanized for reasons unrelated to this study, which was approved by the Ethical Committee of Utrecht University. The dogs were between 18 and 48 months of age (median 23 months), two were mongrels and six were beagles. One dog was female and seven were male, all of the dogs were sexually intact.

Primary cell culture

The adrenocortical cell suspensions were prepared as described previously.[20] In short, the adrenal cortices were digested in a collagenase solution, then filtered and washed. The cell

suspensions were diluted to $1x10^5$ cells/mL with DMEM F-12 (Gibco, Invitrogen, Breda, The Netherlands) with 1% Insulin-Transferrin-Selenium (Gibco), 0.125% BSA, 2.5% Nu-Serum (Corning, Amsterdam, The Netherlands) and 1% penicillin/streptomycin, and seeded in Multiwell 96 well plates (100 µL per well) (Primaria™, Corning).

Two 96 well plates were used for each adrenal cell suspension: one plate for cortisol/DNA ratio measurements and one plate for reverse transcription quantitative PCR (RT-qPCR) analysis. The cells were left to attach for 4 to 7 days at 37 °C in a humidified atmosphere of 95% air and 5% CO_2, after which the culture medium was refreshed prior to compound incubations.

Stock solutions of 500 µM were prepared for compounds #776 and #299, dissolved in 10 mM HCl. The cells were incubated with 50 nM ACTH (1-24) (Synacthen®, Sigma-tau BV, Utrecht, The Netherlands) and 50 nM, 500 nM and 5 µM of #776 and #299. To determine whether the compounds would only affect cortisol production when ACTH(1-24) was added, cells were also incubated without ACTH(1-24) and with 5 µM of #776 and #299. Incubations were performed in quadruplicate. After 24 hours of incubation, cortisol concentrations were measured in the culture medium of four wells per condition by radioimmunoassay as described previously.[21]

Table 1 Primers used for RT-qPCR

Target gene	Primer sequence (5' > 3')	Product size (bp)	Annealing Tm (°C)
StAR	Fw: CTC TGC TTG GTT CTC GG Rv: CCT TCT TCC AGC CTT CC	125	62.5
CYP11A1	Fw: CAC CGC CTC CTT AAA AAG TAA CAA G Rv: GCT GCG TGC CAT CTC GTA G	129	*63.3*
CYP17A1	Fw: CCT GCG GCC CCT ATG CTC Rv: GGC CGG TAC CAC TCC TTC TCA	134	60.0
HSD3B2	Fw: CAG GAG GGT TTC TGG GTC AG Rv: AGG CTC TCT TCA GGC ACT GC	186	56.5
CYP21A2	Fw: AGC CCG ACC TTC CCC TCC ACC TG Rv: TCT GCC GGC GAA GTC CAC CCA TTT	152	64.5
CYP11B1	Fw: GCC TAC CCC TTG TGG ATG AC Rv: CTC TGT GAC TGC TGT CTG GG	126	62.0
MC2R	Fw: TCA TGT GGT TTT GCC GGA AGA GAT Rv: AAT GGC CAG GCT GCA AAT GAA A	138	58.5
MRAP	Fw: CAC AGG TGA GGA ACA ACG Rv: ATC GAA GGT CAG TCC TGG	227	64.6
RPS19	Fw: CCT TCC TCA AAA AGT CTG GG Rv: GTT CTC ATC GTA GGG AGC AAG	95	61.0
SDHA	Fw: GCC TTG GAT CTC TTG ATG GA Rv: TTC TTG GCT CTT ATG CGA TG	92	61.0
HPRT	Fw: AGC TTG CTG GRG AAA AGG AC Rv: TTA TAG TCA AGG GCA TAT CC	104	58.0
YWHAZ	Fw: CGA AGT TGC TGC TGG TGA Rv: TTG CAT TTC CTT TTT GCT GA	94	58.0

DNA measurements

To correct for differences in number of cells per well, DNA was measured in each well to calculate cortisol/DNA ratios. After removing the culture medium, the culture plates underwent three freeze/thaw cycles, after which 50 µL Tris/EDTA (10 mM Tris, 1 mM EDTA, pH 8.0) was added to each well. The Qubit® dsDNA HS Assay Kit (Fisher Scientific, Landsmeer, The Netherlands) was used according to the manufacturer's instructions and DNA concentrations were measured with the Qubit® 2.0 Fluorometer (Fisher Scientific). The cortisol/DNA ratios were calculated of four wells per condition, of which the results were averaged prior to statistical analysis.

RT-qPCR

After removing the culture medium, the wells for each condition were pooled and RNA was isolated from the cells with the RNeasy Micro Kit (QIAGEN, Venlo, The Netherlands), including the DNase treatment, according to the manufacturer's instructions. RNA concentrations were measured with NanoDrop (ND-1000, Isogen Life Science, Utrecht, The Netherlands), after which cDNA was synthesized from 500 ng total RNA with the iScript™ cDNA Synthesis Kit (Bio-Rad) according to protocol. The cDNA was subsequently diluted to 1 ng/µL. RT-qPCR analysis was used to determine the mRNA expression of eight genes: steroidogenic acute regulatory protein (*StAR*), cytochrome P450 side chain cleavage (*CYP11A1*), *HSD3B2*, 17α-hydroxylase/17,20-lyase (*CYP17A1*), 21-hydroxylase (*CYP21A2*), 11β-hydroxylase (*CYP11B1*), *MC2R* and *MRAP* (primers shown in Table 1). Optimization and confirmation of the primer specificity were performed as described previously.[22]

To correct for differences in cDNA concentrations, Ribosomal protein S19 (RPS19), succinate dehydrogenase complex subunit A (*SDHA*), hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*) were used as reference genes (primers shown in Table 1).[23,24]

SYBRgreen supermix (Bio-Rad) was used for the RT-qPCR reactions, and amplification was performed using a CFX 384 Touch™ Real-Time PCR Detection System (Bio-Rad) with the following cycle parameters: initial denaturation for 3 min at 95 °C, then 40 cycles of 10 s at 95 °C followed by 30 s at the primer-specific optimal annealing temperature, and ending with melting curve analysis by one cycle of 10 s 95 °C and a temperature increment of 0.5 °C for 5 s from 65 °C to 95 °C. To exclude the possibility of interfering genomic DNA, for each sample a control where no reverse transcriptase was added in the cDNA reaction was analyzed. Data were analyzed with CFX Manager 3.1 (Bio-Rad). Two technical replicates were used for each sample. GeNorm software[25] was used to analyze expression levels of the reference genes, which justified their use. To calculate the normalized relative expression of each target gene, the $2^{-\Delta\Delta Ct}$ method[26] was used.

Statistical analysis

Logarithmic transformation resulted in normally distributed data, which was confirmed with the Shapiro-Wilk test. After logarithmic transformation, cortisol/DNA ratios and RT-qPCR fold changes were analyzed with repeated measures ANOVA with a Bonferroni post-hoc test to correct for multiple comparisons. A P-value of < 0.05 was considered significant. Data are reported as mean ± SEM of eight individual cell cultures.

Results

Cortisol production: cortisol/DNA ratios

Incubation with 50 nM ACTH(1-24) increased the cortisol/DNA ratio 35.4 ± 10.4-fold ($P < 0.0001$). Co-incubation with #776 dose-dependently inhibited the ACTH-stimulated cortisol/DNA ratio by 33.5 ± 7.1% at 500 nM and by 38.0 ± 5.2% at 5 μM (Fig. 1A). Co-incubation with #299 dose-dependently inhibited the ACTH-stimulated cortisol/DNA ratio by 25.1 ± 5.0% at 50 nM, by 78.8 ± 7.2% at 500 nM and by 90.7 ± 2.3% at 5 μM (Fig. 1A). In the non-ACTH-stimulated cells, neither compound inhibited the cortisol/DNA ratio. On the contrary, #776 slightly but significantly ($P = 0.002$) increased the non-ACTH-stimulated cortisol/DNA ratio 1.4 ± 0.1-fold at 5 μM (Fig. 1B). Compound #299 did not affect the non-ACTH-stimulated cortisol/DNA ratio (1.1 ± 0.1-fold, $P = 1$) (Fig. 1B).

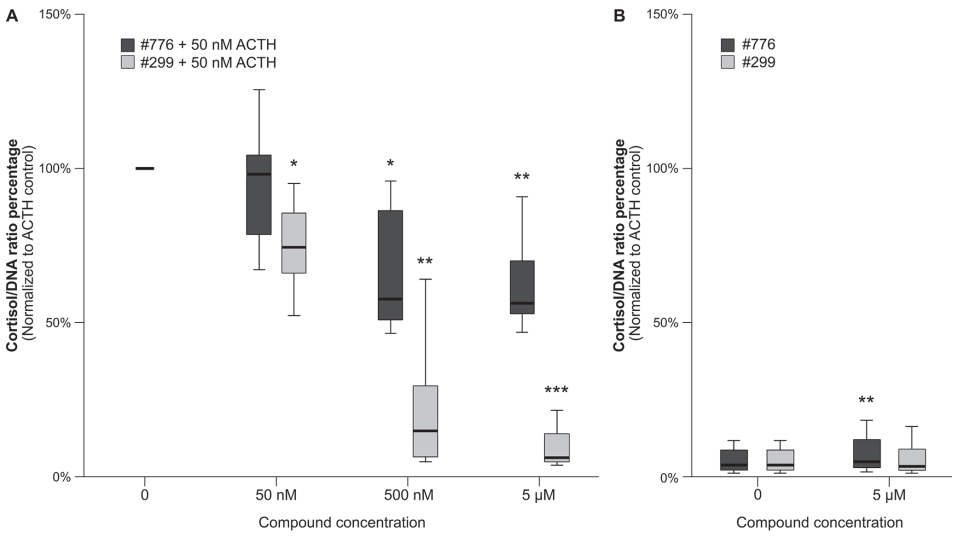

Figure 1 The effects of compounds BIM-22776 (#776) and BIM-22A299 (#299) on the cortisol production of ACTH(1–24)-stimulated (A) and non-ACTH-stimulated (B) canine primary adrenocortical cell cultures (n = 8). Cortisol/DNA ratios are shown in percentages, normalized to the ACTH-stimulated control. Asterisks represent significant differences compared to the ACTH-stimulated controls: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

RT-qPCR

Incubation with 50 nM ACTH(1-24) significantly ($P < 0.01$ or lower) upregulated the mRNA expression of all the genes analyzed in this study, but most notably that of *CYP17A1*, followed by *MRAP, CYP11B1* and *StAR* (Fig. 2). Co-incubation with 5 μM #776 significantly inhibited the ACTH-stimulated expression of five of the eight genes analyzed in this study (Fig. 2), while co-incubation with 5 μM #299 significantly inhibited the ACTH-stimulated expression of all the genes analyzed in this study (Fig. 2).

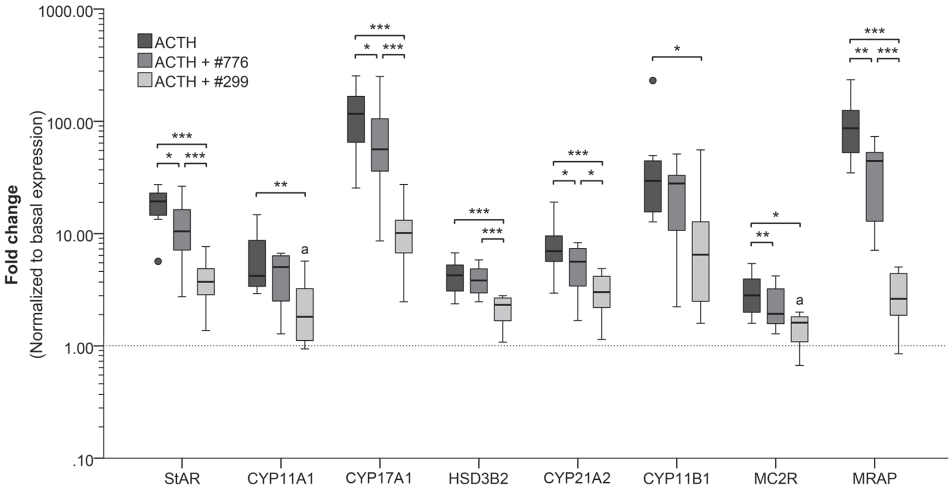

Figure 2 The effects of incubation with 50 nM ACTH(1–24) and of coincubation of ACTH(1–24) with 5 μM of compounds BIM-22776 (#776) and BIM-22A299 (#299) on the relative mRNA expression of steroidogenic enzymes, MC2R, and MRAP in canine primary adrenocortical cell cultures (n = 8). Fold changes are normalized to the non-ACTH stimulated controls, i.e. the basal expression. Asterisks represent significant differences: *P < 0.05, **P < 0.01, ***P < 0.001. All conditions were significantly different from the basal expression, except when indicated with an "a". StAR, steroidogenic acute regulatory protein; *CYP11A1,* cytochrome P450 side chain cleavage; *CYP17A1*, 17α-hydroxylase/17,20-lyase; *HSD3B2*, 3β-hydroxysteroid hydrogenase type 2; *CYP21A2*, 21-hydroxylase; *CYP11B1*, 11β-hydroxylase; *MC2R*, melanocortin 2 receptor; *MRAP*, melanocortin type 2 accessory protein.

Discussion

The results of this study show that canine primary adrenocortical cell culture stimulated with synthetic ACTH(1-24) is a functional *in vitro* model to test the efficacy of MC2R antagonists. Moreover, this study shows that #299 and #776 are effective MC2R antagonists, of which #299 is the most potent.

Multiple attempts to create or isolate MC2R antagonists have been made previously,[27–29] mostly with varying effects. Recently, Bouw *et al.* (2014) showed that GPS1573 and GPS1574, two ACTH analogs, can antagonize MC2R *in vitro* in the nanomolar range in a human embryonic kidney cell line transfected with the MC2R.[30] However, a subsequent study by Nensey *et al.* (2016) demonstrated that GPS1573 could not antagonize the adrenal response to ACTH in neonatal rats *in vivo*. High concentrations of GPS1574 did dose-dependently inhibit corticosterone production in these rats.[31] Whether #776 and #299 can antagonize the adrenal response to ACTH *in vivo* remains to be determined, but using primary adrenocortical cell cultures might be a better predictor of *in vivo* functionality than using homogeneous and genetically altered cell lines from extra-adrenal sources.

In this study we evaluated how the compounds affected the cortisol production of both ACTH-stimulated and non-ACTH-stimulated cells. We aimed to mimic ACTH-dependent hypercortisolism by adding 50 nM synthetic ACTH(1-24). This ACTH concentration significantly and strongly increased the cortisol production, which indicates that the cells responded as expected and that canine primary adrenocortical cell culture is a good *in vitro* model to test the effects of ACTH. Because we corrected the cortisol values with the DNA concentrations, we could exclude the possibility that any observed differences in the cortisol production were caused by a difference in the number of cells.

In the non-ACTH-stimulated canine adrenocortical cells, incubation with #776 slightly but significantly increased the cortisol production, which could indicate that #776 has agonistic properties when the natural agonist is absent. Since using MC2R antagonists in a clinical setting would only be indicated when ACTH is excessively secreted, this phenomenon is expected to be clinically irrelevant. Incubation with #299 did not affect non-ACTH-stimulated cortisol production.

To evaluate whether the compounds were able to antagonize the ACTH-induced changes in the mRNA expressions of steroidogenic enzymes, the *MC2R* and *MRAP*, we performed RT-qPCR analyses. ACTH upregulated the mRNA expressions of all the genes analyzed in this study, while #299 inhibited the ACTH-stimulated mRNA expressions of these genes. These results show that #299 can antagonize the ACTH-induced changes in the mRNA expressions of steroidogenic enzymes, the *MC2R* and *MRAP*. Co-incubation with #776 downregulated the ACTH-stimulated mRNA expression of most of the genes analyzed in this study, but not of all genes and not as vigorously as #299.

One of the advantages of using MC2R antagonists is that functional MC2R expression is limited to the adrenal gland; antagonism of the MC2R is therefore unlikely to result in off-target effects. However, since the other melanocortin receptors have a variety of functions in other tissue types, inadvertently antagonizing or agonizing these receptors could result in many unwanted side-effects.[12] It is therefore important to determine whether the compounds are selective for the MC2R, and do not affect the other melanocortin receptors. This compound selectivity will have to be determined in future studies.

Conclusion

In conclusion, the results of this study indicate that canine primary cell culture is a valuable *in vitro* system to test MC2R antagonists, and that these compounds, but especially #299, are effective MC2R antagonists *in vitro*. To determine their efficacy *in vivo*, further studies are warranted. Antagonism of the MC2R is a promising potential treatment approach in canine PDH.

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Chapter

6

Steroidogenic Factor-1 Inverse Agonists as a Treatment Option for Canine Hypercortisolism: *In Vitro* Study

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Abstract

Hypercortisolism is one of the most commonly diagnosed endocrinopathies in dogs, and new targeted medical treatment options are desirable. Steroidogenic factor-1 (SF-1), an orphan nuclear hormone receptor, is a key regulator of adrenal steroidogenesis, development, and growth. In pituitary-dependent hypercortisolism (PDH), high plasma ACTH concentrations increase the transcriptional activity of SF-1. In adrenal-dependent hypercortisolism, SF-1 expression is significantly greater in dogs with recurrence after adrenalectomy than in those without recurrence. Inhibition of SF-1 could therefore be an interesting treatment option in canine spontaneous hypercortisolism. We determined the effects of 3 SF-1 inverse agonists, compounds IsoQ A, #31, and #32, on cortisol production, on the messenger RNA (mRNA) expression of steroidogenic enzymes and steroidogenic factors, and on cell viability, in primary adrenocortical cell cultures of 8 normal adrenal glands and of 3 cortisol-secreting adrenocortical tumors (ACTs). To mimic PDH, the normal adrenocortical cell cultures were stimulated with ACTH. The results show that only compound #31 inhibited cortisol production and SF-1 target gene expression in non–ACTH-stimulated and ACTH-stimulated normal adrenocortical cells but did not affect cell viability. In the ACT cell cultures, the effects of #31 on cortisol production and target gene expression were variable, possibly caused by a difference in the *SF-1* mRNA expressions of the primary tumors. In conclusion, inhibition of SF-1 activity shows much promise as a future treatment for canine hypercortisolism.

Introduction

In dogs, one of the most frequently diagnosed endocrine disorders is hypercortisolism (Cushing's syndrome). Spontaneous hypercortisolism is caused by an ACTH-secreting pituitary adenoma (pituitary-dependent hypercortisolism [PDH]) in 80% to 85% of cases and by a primary functional cortisol-secreting adrenocortical tumor (ACT) in 15% to 20% of cases, of which the majority is an adrenocortical carcinoma.[1,2]

For medical treatment of spontaneous canine hypercortisolism, the adrenocorticolytic drug mitotane (o,p'-DDD) and the adrenal enzyme inhibitor trilostane are used most often. Mitotane destroys adrenocortical cells, but because it can cause serious side effects,[3–5]

its use has been largely replaced by trilostane. Trilostane competitively inhibits the steroidogenic enzyme 3β-hydroxysteroid dehydrogenase type 2 (HSD3B2) and thereby inhibits cortisol production. The drawbacks of treatment with trilostane include disturbance of the renin-angiotensin-aldosterone axis, and possibly occurrence of apoptosis and necrosis in the adrenal cortex.[6–8] Moreover, trilostane does not affect the growth of an ACT or its metastases.[5,9]

To improve the medical treatment of dogs with spontaneous hypercortisolism, new targeted medical treatment is desirable. For this new medical treatment, an interesting target could be steroidogenic factor-1 (SF-1/Ad4BP; NR5A1), which is an orphan nuclear receptor that regulates adrenal steroidogenesis, development, and growth.[10] Steroidogenic factor-1 was initially discovered as a transcription factor for genes encoding steroidogenic enzymes.[11,12] This transcriptional activity of SF-1 can be stimulated by the binding of ACTH to the melanocortin 2 receptor (MC2R) in the adrenal cortex;[13] therefore, increased SF-1 activity is an important characteristic of PDH. Steroidogenic factor-1 was later discovered to be essential also in adrenal development. Mice with targeted disruption of the SF-1 gene (SF-1$^{-/-}$ mice) were born without adrenal glands and gonads and died shortly after birth.[14,15] Not only the presence or absence but also the dose of SF-1 is important.[16–18] Greater SF-1 dosages increased proliferation in the human adrenocortical carcinoma cell line NCI-H295R and induced adrenocortical neoplasia in mice.[19] Furthermore, *SF-1* messenger RNA (mRNA) expression was significantly greater in dogs with ACTs that had recurrence of hypercortisolism within 2.5 yr after adrenalectomy than in dogs that had no recurrence for at least 2.5 yr after adrenalectomy.[20] Taken together, these data suggest that inhibition of SF-1 activity might lead to inhibition of steroidogenesis and inhibition of ACT growth.

Previous studies have focused on identifying compounds that can inhibit human SF-1 activity. One such study, using ultra-high-throughput screening, identified 2 isoquinolinone analogs, SID7969543 (IsoQ A) and SID7970631 (IsoQ B), as selective SF-1 inverse agonists.[21] Identification of these compounds led to the development of analogs with improved SF-1 inhibitor potency, lower cellular toxicity, and improved selectivity: compounds #31 and #32.[22] Doghman *et al.*[23] demonstrated that, in conditions of increased SF-1 expression, compounds IsoQ A, #31, and #32 selectively decreased forskolin-stimulated steroid hormone production and inhibited NCI-H295 R cell proliferation.

Based on the importance of SF-1 in adrenal steroidogenesis and proliferation, inhibition of SF-1 activity could be an interesting treatment option in canine hypercortisolism. This study is the first to investigate the effects of SF-1 inverse agonists in canine primary adrenocortical cell culture of normal adrenals, stimulated with synthetic ACTH to mimic PDH, and of ACTs.

Materials and methods

Animals and tissues

For primary cell cultures, the adrenal glands of 8 healthy dogs were used. These dogs were euthanized for reasons unrelated to the present study, which was approved by the Ethical Committee of Utrecht University. The dogs were aged between 14 and 62 mo (median 35 mo) and weighed between 10 and 29 kg (median 16 kg). Four dogs were of mixed breed and 4 dogs were Beagles. Two dogs were female and 6 were male, all dogs were intact.

The cortisol-producing ACTs were retrieved after unilateral adrenalectomy of 3 dogs. The dogs were aged between 98 and 134 mo (median 118 mo) and weighed between 10 and 18 kg (median 13 kg). One dog was an intact male Fox Terrier, one dog was an intact female Västgötaspets, and one dog was male, castrated, and of mixed breed. The suspicion of hypercortisolism was based on the medical history and the findings on physical examination. The diagnosis of a cortisol-producing ACT was confirmed by the finding of nonsuppressible hypercortisolism with endocrine testing, combined with the demonstration of an ACT with an atrophic contralateral adrenal gland by ultrasonography or computed tomography.[24] The dogs did not receive any drugs to inhibit cortisol production prior to the adrenalectomy. Histopathological evaluation confirmed the diagnosis of an ACT in all dogs. The ACTs were classified by a single pathologist based on the criteria described by Labelle *et al.*,[2] which classified 2 ACTs as carcinomas and one ACT as an adenoma.

For reverse transcriptase quantitative PCR (RT-qPCR) analysis of primary tissues, the aforementioned ACTs and the adrenal glands of 4 healthy dogs were used. The healthy dogs aged between 14 and 58 mo (median 24 mo) and weighed between 10 and 26 kg (median 23 kg). Three dogs were of mixed breed, and one dog was a Beagle. Three dogs were female and one was male, all dogs were intact.

Cell culture

The adrenal glands were collected in ice-cold Hanks Balanced Salt Solution (Gibco, Invitrogen, Merelbeke, Belgium) and cleared of surrounding tissues. Normal adrenals were cut in half length-wise to scrape out the medulla. In the case of the ACTs, only apparent tumorous tissue was used.

The adrenal tissues were cut into pieces and digested for 60 to 75 min at 37°C in a mixture of Leibowitz L15 (Gibco, Invitrogen, Breda, The Netherlands), 3mg/mL collagenase 1A (Sigma-Aldrich, Zwijndrecht, The Netherlands), 0.05 mg/mL DNAse (Sigma-Aldrich), 20mM D-(+)-Glucosemonohydrate (Sigma-Aldrich), 0.2% bovine serum albumin (BSA; Sigma-Aldrich), and 1% penicillin/streptomycin (Gibco). The digested tissue solutions were filtered through 100 and 70 µm EASYstrainer filters (Greiner Bio-One, Alphen aan den Rijn, The Netherlands) and subsequentlymixed in a 1:1 ratio with Leibowitz L15 containing 20% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin. A 3% BSA-cushion was pipetted at the bottomof the tube to remove cell debris, after which the tissue

solutions were centrifuged at 190 × g for 10 min at 4°C. The pellets were washed in Leibowitz L15 containing 10% FBS and 1% penicillin/streptomycin and again centrifuged. The cells were counted with a Bürker Türk counting chamber and diluted to 1×10^5 cells/mL with Dulbecco's Modified Eagle Medium F-12 (Gibco) containing 1% Insulin-Transferrin-Selenium (Gibco), 0.125% BSA, 2.5% Nu-Serum (Corning, Amsterdam, The Netherlands), and 1% penicillin/streptomycin. The cells were seeded in Multiwell 96 well plates (1×10^4 cells per well, Primaria, Corning) to measure cortisol and DNA concentrations and cell viability, and in Multiwell 24 well plates (1×10^5 cells per well) to isolate RNA for RT-qPCR analysis.

The cells were left to attach for 4 to 7 d at 37°C in a humidified atmosphere of 95% air and 5% CO_2; then, the culture medium was refreshed before the cells were incubated with the compounds.

Chemicals

Three compounds were used: compound IsoQ A (SID7969543, Sigma-Aldrich, Zwijndrecht, The Netherlands), compound #31 (F1808-0154, Life Chemicals, Kiev, Ukraine), and compound #32 (F1808-0165, Life Chemicals). The compounds were dissolved in dimethyl sulfoxide (DMSO; Merck KGaA, Darmstadt, Germany) at 20 mM, then aliquoted and stored at 20°C.

The cells were incubated with 10 nM, 100 nM, 1 μM, and 10 μM of the compounds, and with (normal adrenocortical cells) and without (normal adrenocortical and ACT cells) 1 μM ACTH [1–24] (Synacthen, Novartis Pharma BV, Arnhem, The Netherlands). The DMSO end concentration was 0.05% in all conditions. Incubations were performed in quadruplicate in the 96-well plates and in duplicate in the 24-well plates. After 72 h of incubation, the culture medium was removed and used to measure cortisol concentrations by RIA as described previously.[25]

DNA measurements

After the culture medium was removed, the culture plates underwent 3 freeze/thaw cycles. Thereafter, 50 mL Tris/EDTA (10 mM Tris, 1 mM EDTA, pH 8.0) was added to each well, after which the Qubit dsDNA HS Assay Kit (Fisher Scientific) was used according to the manufacturer's instructions. Deoxyribonucleic acid concentrations were measured with a Qubit 2.0 Fluorometer (Fischer Scientific).

Viability

After 72 h of incubation, 10 μL of alamarBlue reagent (Thermo Fisher) was added to each well in 96-well plates. The cells were incubated with the alamarBlue reagent for 4 h at 37°C in the dark, after which fluorescence was measured on a DTX 880 Multimode Detector (Beckman Coulter, Woerden, The Netherlands) at 535 nm excitation, 595 nm emission.

Reverse transcriptase quantitative PCR

For RT-qPCR analysis of the primary ACTs, tissue fragments were snap frozen in liquid nitrogen within 10 to 20 min after surgical removal and RNA was isolated with the RNeasy Mini Kit (QIAGEN, Venlo, The Netherlands) according to the manufacturer's instructions.

For RT-qPCR analysis of cultured cells, the culture medium was removed from the 24-well plates after 72 h of incubation with compounds and RNA was isolated from the cells with the RNeasy Micro Kit (QIAGEN), including a DNAse (QIAGEN) step to avoid genomic contamination, according to the manufacturer's instructions. The RNA concentrations were measured with NanoDrop (ND-1000, Isogen Life Science, Utrecht, The Netherlands). The iScript cDNA Synthesis Kit (Bio-Rad) was used according to the manufacturer's instructions to synthesize complementary DNA (cDNA), which was subsequently diluted to 1 ng/mL.

Reverse transcriptase quantitative PCR analysis was used to determine the mRNA expression of 3 steroidogenic enzymes and 3 steroidogenic factors. The steroidogenic enzymes that were analyzed were 17α-hydroxylase/17,20-lyase (*CYP17A1*), *HSD3B2*, and 21-hydroxylase (*CYP21A2*). The steroidogenic factors thatwere analyzed were *SF-1*, dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome gene 1 (*DAX1*), and *MC2R*. Primers used for *CYP17A1, HSD3B2, CYP21A2, MC2R*, and *SF-1* have been described previously.[26] New primers were designed for *DAX1* (forward 5'-CTTTAACCCGGACCTGC-3', reverse 5'-GCCTGAAGAATAGCTCCAC-3'). The primer products were sequenced using an ABI3130XL Genetic analyzer to confirm the correct identity of the transcript. To correct for differences in cDNA concentrations, the reference genes that were analyzed were ribosomal protein S5, ribosomal protein S19, signal recognition particle receptor, and hypoxanthine-guanine phosphoribosyltransferase.[27,28]

The RT-qPCR reactions were performed using SYBRgreen supermix (Bio-Rad) in a CFX384 Touch Real-Time PCR Detection System (Bio-Rad) instrument. To analyze the expression levels of the reference genes, GeNorm software[29] was used, which justified their use. To calculate the normalized relative mRNA expression of each target gene, the $2^{-\Delta\Delta Ct}$-method[30] was used.

Statistical analysis

Normally distributed data (cortisol and DNA data) were analyzed with repeated measures analysis of variance with a post hoc Bonferroni correction. Sphericity was checked using Mauchly's test, and the Greenhouse-Geisser correction was used when the assumption of sphericity was violated. Nonnormally distributed data (alamarBlue and RT-qPCR data) were analyzed with the Friedman test. Significant outcomes were analyzed post hoc with the Wilcoxon signed-rank test with a Bonferroni correction. P-values of < 0.05 were con-

sidered significant. Cortisol data are reported relative to the DMSO vehicle control in mean standard error of the mean.

Results

Cortisol production, DNA content, and cell viability

Normal adrenocortical cell cultures (n = 8)

In the normal adrenocortical cell cultures, 10 μM of #31 significantly inhibited the cortisol production of the non–ACTH-stimulated cells by 55 ± 10% (P = 0.026) (Fig. 1A) and of the ACTH-stimulated cells by 57 ± 5% (P = 0.002) (Fig. 1B). Compounds IsoQ A and #32 did not significantly affect the cortisol production of either non–ACTH-stimulated cells (Fig. 1A) or ACTH-stimulated cells (Fig. 1B).

None of the compounds affected either the DNA content per well or the resazurin reduction in the alamarBlue viability assay of either non–ACTH-stimulated or ACTH-stimulated normal adrenocortical cells (data not shown).

Adrenocortical tumor cell cultures (n = 3)

In the ACT cell cultures, 10 mM of #31 inhibited the cortisol production of 2 ACTs but did not inhibit cortisol production of one ACT (Table 1). Compound IsoQ A slightly inhibited the cortisol production of only one AT, and #32 did not inhibit the cortisol production of any ACT (Table 1). None of the compounds affected either the DNA content per well or the resazurin reduction in the alamarBlue viability assay of the ACT cells (data not shown).

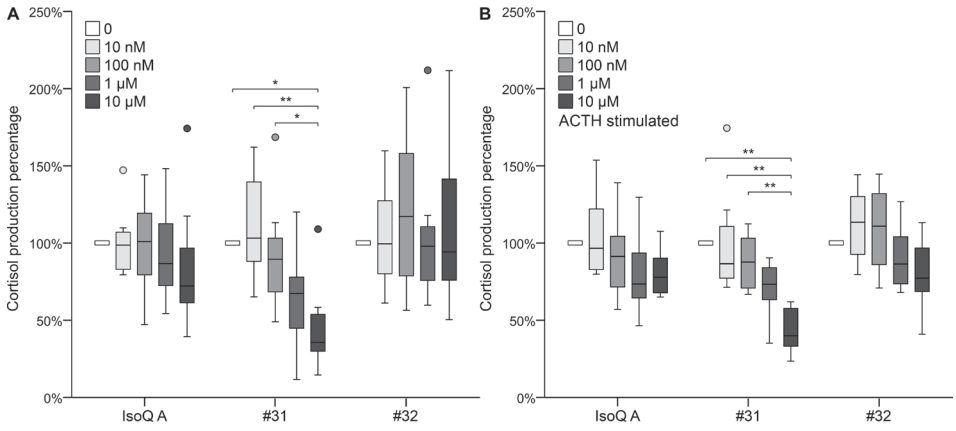

Figure 1 The effects of compounds IsoQ A, #31, and #32 on the cortisol production of non–ACTH-stimulated (A) and ACTH-stimulated (B) canine primary normal adrenocortical cell cultures (n = 8). Cortisol concentrations are shown in percentages, normalized to the control. Asterisks represent significant differences compared to the control: *P < 0.05 and **P < 0.01.

Table 1 The cortisol production of ACT cell cultures compared to the control and the *SF-1* mRNA expression of the primary ACT cells.

ACT	% Cortisol production + IsoQ A	% Cortisol production + #31	% Cortisol production + #32	*SF-1* fold change
1	84	106	179	0.8
2	107	85	144	1.7
3	112	66	108	4.1

The percentage of cortisol production compared to the DMSO vehicle control after incubation with 10 μM of compounds IsoQ A, #31, or #32 in each individual canine cortisol-secreting ACT cell culture and the fold change of *SF-1* in the primary ACT, relative to 4 normal adrenal glands. ACT, cortisol-secreting adrenocortical tumor; *SF-1*, steroidogenic factor-1.

Reverse transcriptase quantitative PCR

Normal adrenocortical cell cultures (n = 8)

In the normal adrenocortical cell cultures, addition of 1 mM ACTH (1–24) significantly upregulated the mRNA expression of *CYP17A1* (P = 0.012), *HSD3B2* (P = 0.012), *CYP21A2* (P = 0.012), *SF-1* (P = 0.017) and *MC2R* (P= 0.012), whereas it significantly downregulated the mRNA expression of *DAX1* (P = 0.012) (Fig. 2).

In the non–ACTH-stimulated normal adrenocortical cell cultures, #31 significantly downregulated the mRNA expression of *CYP17A1* (P = 0.035), *HSD3B2* (P = 0.035), *CYP21A2* (P = 0.035) (Fig. 3A), and *MC2R* (P = 0.035) (Fig. 3B). In the ACTH-stimulated normal adrenocortical cell cultures, #31 significantly downregulated the expression of *HSD3B2* (P = 0.035), *CYP21A2* (P = 0.035) (Fig. 3C), and *MC2R* (P = 0.035) (Fig. 3D). Compounds IsoQ A and #32 did not affect the mRNA expression of the genes analyzed in this study in either non–ACTH-stimulated or ACTH-stimulated normal adrenocortical cell cultures (Fig. 3).

Adrenocortical tumor cell cultures (n = 3)

Based on the results from the normal adrenocortical cell cultures, we determined whether #31 had similar effects on the mRNA expressions of *CYP17A1, HSD3B2, CYP21A2,* and *MC2R* in the ACT cell cultures. In all the ACT cell cultures, #31 inhibited the expression of *CYP17A1* (fold changes 0.4, 0.7, and 0.7), *HSD3B2* (fold changes 0.3, 05, and 0.9), *CYP21A2* (fold changes 0.3, 0.7, and 0.8) and *MC2R* (0.4, 0.4, 0.5). Due to the low number of ACT cell cultures, no statistical analyses were performed on this data. The fold changes of *SF-1* in the primary ACTs relative to 4 normal adrenal glands are shown in Table 1, combined with the percentages of cortisol production compared to the DMSO vehicle control when incubated with 10 μM of compounds IsoQ A, #31 and #32.

Discussion

This study demonstrates that canine primary adrenocortical cell culture is a valuable *in vitro* system that can be used to test potential future treatment options for canine hypercortisolism. We have shown that, with our culture protocol, canine primary adrenocortical cells retained their hormone-producing abilities and their responsiveness to ACTH *in vitro*. This makes this *in vitro* model superior to the most commonly used human ACT cell line NCI-H295R, which responds only mildly or not at all to ACTH stimulation[31] and is therefore not a suitable model to study PDH. Furthermore, the 3 ACT cell cultures provide a proof of concept that this primary culture system is not only possible for normal canine adrenocortical cells but also for canine ACT cells. Because of the heterogeneous nature of cells in primary cell culture, this *in vitro* system more closely resembles the heterogeneous *in vivo* conditions than a single cell line does and could also be used as an animal *in vitro* model for human hypercortisolism.

This study shows that compound #31 is an effective inhibitor of cortisol production and SF-1 target gene expression in primary cell culture of normal canine adrenocortical cells, both with and without ACTH stimulation. The inhibition of cortisol production does not seem to be caused by a decrease in the number or the viability of the cells, because #31 did not decrease the DNA content or inhibit the cell viability. Compound #31 was more potent than IsoQ A or #32, both in terms of inhibition of mRNA expression levels of SF-1 target genes and of inhibition of cortisol production. This difference in potency makes #31 an interesting candidate for further development into a new drug to treat canine hypercortisolism.

The objective of using SF-1 inverse agonists is to inhibit the transcriptional activity of SF-1. The activity of SF-1 is affected by posttranslational modifications such as

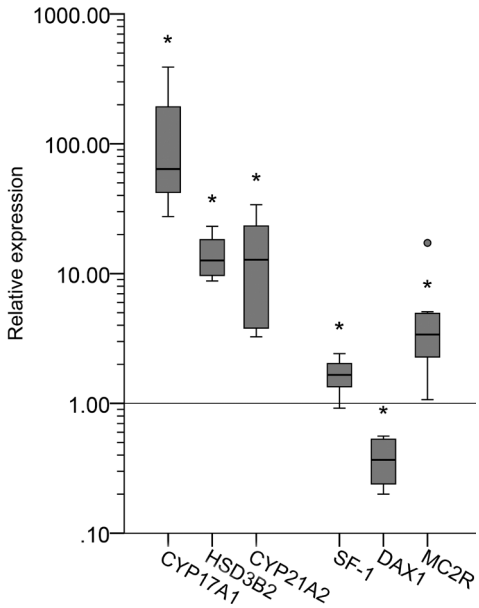

Figure 2 The effects of addition of 1 μM ACTH[1–24] on the relative mRNA expression of steroidogenic enzymes (*CYP17A1, HSD3B2*, and *CYP21A2*) and steroidogenic factors (*SF-1, DAX1*, and *MC2R*) in canine normal adrenocortical cell cultures (n = 8). Fold changes are normalized to the non–ACTH-stimulated control. Asterisks represent significant differences compared to the non–ACTH-stimulated controls: *P < 0.05. *CYP17A1,* 17α-hydroxylase/17,20-lyase; *CYP21A2*, 21-hydroxylase; *DAX1*, dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome gene 1; *HSD3B2*, 3β-hydroxysteroid dehydrogenase 2; *MC2R,* melanocortin 2 receptor; *SF-1,* steroidogenic factor-1.

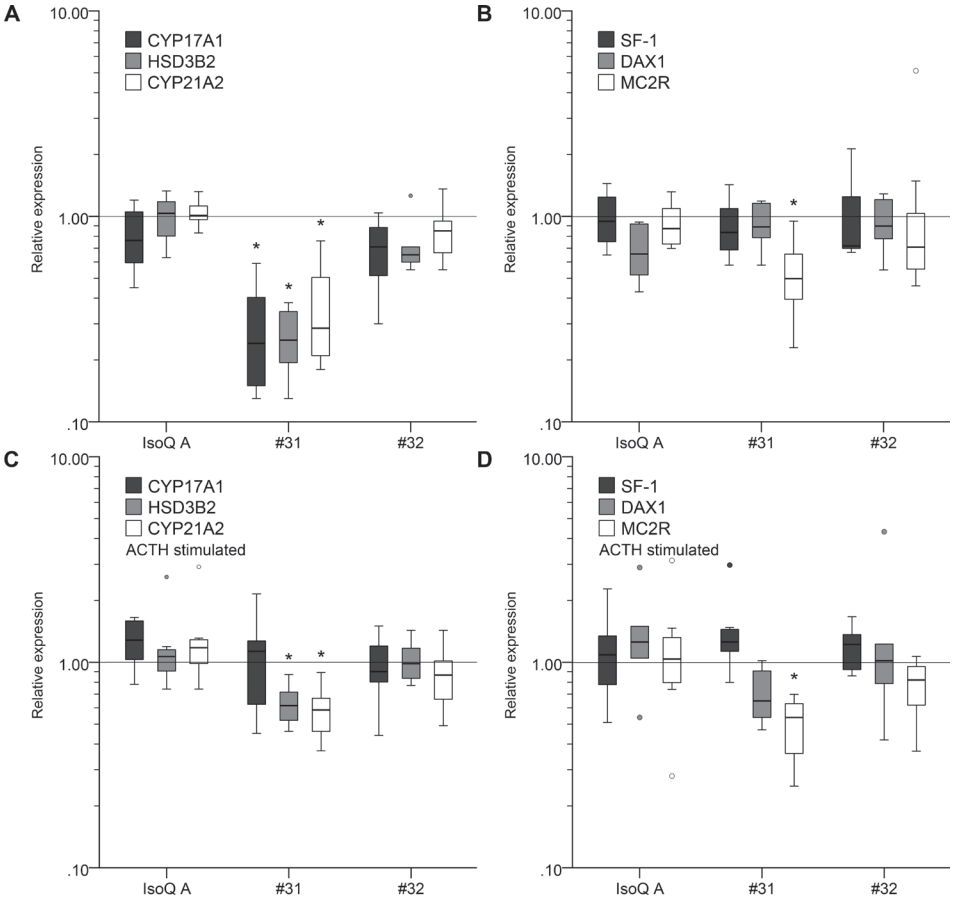

Figure 3 The effects of compounds IsoQ A, #31, and #32 on the relative messenger RNA expression of steroidogenic enzymes (A and C) and steroidogenic factors (B and D) in non–ACTH-stimulated (A and B) and ACTH-stimulated (C and D) canine primary normal adrenocortical cell cultures (n = 8). Fold changes are normalized to thecontrols. Asterisks represent significant differences compared to the ACTH-stimulated controls: *P < 0.05. *CYP17A1,* 17α-hydroxylase/17,20-lyase; *CYP21A2*, 21-hydroxylase; *DAX1*, dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome gene 1; *HSD3B2,* 3β-hydroxysteroid dehydrogenase 2; *MC2R,* melanocortin 2 receptor; *SF-1,* Steroidogenic factor-1.

phosphorylation, acetylation, and sumoylation.[13,32–34] Although the term "orphan nuclear receptor" implies that it is unknown whether there are physiological ligands that regulate the transcriptional activity of SF-1, more recent structural analyses showed that bacterially expressed SF-1 protein contains a large hydrophobic ligand-binding pocket filled with phospholipids, which could function as regulatory ligands.[35–37] Depending on fatty acid tail length, effects of the phospholipids on bacterially expressed SF-1 activity are either inhibitory or stimulatory.[36]

The transcriptional activity of SF-1 can be stimulated by ACTH. Binding of ACTH to the MC2R activates a cyclic AMP (cAMP)-dependent signal transduction cascade that eventually results in increased transcription of steroidogenic enzymes,[13,38] as also shown in Figure 2. This increase in transcription is caused, at least partly, by increased SF-1 activity. In NCI-H295R cells, cAMP signaling activates SF-1 by decreasing the amount of inhibitory ligands bound to SF-1 (such as sphingosine)[39] and increasing the amount of stimulatory ligands bound to SF-1 (such as phosphatidic acid).[40] However, the regulation of nuclear receptors by phospholipids is a relatively young field of research,[41] in which there is still much to be explored.

In addition to increasing SF-1 activity, cAMP signaling also increases SF-1 protein expression.[42] Some studies report that this increased protein expression is not caused by increased mRNA expression of *SF-1* but by increased stability of the SF-1 protein,[42–46] although other studies report that cAMP signaling does increase SF-1 mRNA expression.[47–49] In the present study, we did not determine whether ACTH affected the SF-1 protein expression, but addition of ACTH did significantly increase the SF-1 mRNA expression in the normal canine primary adrenocortical cells. This finding supports the concept that the cAMP-induced increased SF-1 protein expression is at least partly caused by increased *SF-1* mRNA expression.

Another important regulator of the transcriptional activity of SF-1 is DAX1. DAX1 inhibits SF-1 activity both by binding to gene promoters that are regulated by SF-1, as well as by directly interacting with SF-1.[50] Similar to previously reported results,[47] our results show that ACTH inhibits *DAX1* mRNA expression, which shows another way by which ACTH increases SF-1 activity.

How the compounds used in the present study affect the transcriptional activity of SF-1 is still unknown. Possible mechanisms of action include binding inside the ligand binding pocket of SF-1 or interfering with the interaction of transcriptional cofactors with SF-1.[23] In the present study, the compounds did not affect the mRNA expression of *SF-1*, but we did not determine whether the compounds affect the protein expression of SF-1. However, since the compounds did not affect SF-1 protein expression in NCI-H295R cells,[23] it seems unlikely that the effects of the compounds are caused by inhibition of the SF-1 protein expression.

In the present study, #31 resulted in different degrees of cortisol inhibition in the ACT cell cultures, which could be explained by the different *SF-1* mRNA expressions of these tumors. This hypothesis is supported by Table 1, where we see that the adrenocortical cells from the ACT with the greatest SF-1 mRNA expression show most cortisol inhibition by #31, whereas the adrenocortical cells from the AT with the lowest SF-1 mRNA expression show least cortisol inhibition by #31. This is similar to the findings by Doghman *et al.*, who showed that the effect of the compounds is SF-1-dosage dependent,[23] which indicates

that these compounds specifically target SF-1 activity. However, the low number of ACTs used in the present study makes it difficult to draw any reliable conclusions. Nevertheless, we included the 3 ACTs to show that #31 may not only have potential in dogs with PDH but also in dogs with ACTs that have high SF-1 expression.

Since ACTH increases SF-1 activity and the effects of the compounds are SF-1-dosage dependent, we expected that the effects of the compounds would be more evident in the ACTH-stimulated cells. However, the inhibition of cortisol production by #31 seems to be comparable between non–ACTH-stimulated and ACTH-stimulated normal adrenocortical cells. In fact, the inhibition of the mRNA expression of the steroidogenic enzymes by #31 was even greater in the non–ACTH-stimulated cells than in the ACTH-stimulated cells. Possibly, the metabolic stability of the ACTH was greater than that of the compounds, resulting in an underestimation of the effects of the compounds after 72 h of incubation. To confirm this, repeat experiments could include different incubation times, measuring the compound concentrations in the culture medium at different time points or reincubating the cells with fresh compounds during the course of the experiment.

In ACTs, a big advantage of inhibiting SF-1 activity would be to also inhibit ACT cell proliferation. In the study by Doghman *et al.* (2009), compounds IsoQ A, #31, and #32 all significantly inhibited the *SF-1* overexpression–induced cell proliferation in NCI-H295 R cells, which they determined by counting the cells.[23] In the present study, the compounds did not inhibit the DNA content or cell viability of normal adrenocortical or ACT cells after 72 h of incubation. However, the primary adrenocortical cells in our current culture system have a low proliferation rate, and longer incubation times could therefore be necessary to detect an inhibitory effect on cell proliferation. Since our results show that #31 is an effective inhibitor of SF-1–dependent steroidogenesis, the hypothesis that #31 could also inhibit SF-1–dependent proliferation seems likely. To determine whether using SF-1 inverse agonists could be useful to inhibit proliferation of ACTs, further research is necessary on a larger ACT sample size and *in vivo*.

Since ACTH increases SF-1 activity, the use of SF-1 inverse agonists could be beneficial in patients with PDH, which accounts for the majority of dogs with spontaneous hypercortisolism. As previously mentioned, in ACTs, SF-1 inverse agonists will only have additional value in patients of which the ACTs have normal to high *SF-1* expression. Dogs with ACTs with relatively high *SF-1* mRNA expression have a greater chance of recurrence of hypercortisolism compared to dogs with relatively low *SF-1* mRNA expression,[20] similar to humans with ACTs in which SF-1 protein expression is an important prognostic factor.[51,52] This group with high *SF-1* expression therefore has the most need for additional treatment, and SF-1 inverse agonists could be a promising future treatment in these patients. The greatest chance of therapeutic success would therefore be when SF-1 inverse agonists are used in personalized medicine, where the *SF-1* expression of an individual ACT is determined prior to initiation of therapy. Since this can only be determined in

tumors after a patient has undergone adrenalectomy, this could be feasible as postoperative adjuvant therapy. In addition, further research could focus on whether the *SF-1* expression of an ACT can reliably be determined from fine needle biopsies taken from canine ACTs *in situ*. Alternatively, it would be very interesting to study whether there are peripheral biomarkers that correlate with a high *SF-1* expression in the tumor, for example, circulating cell-free microRNAs.[53]

Conclusion

Canine primary adrenocortical cell culture is a valuable *in vitro* culture system that can be used to test potential future treatment options for hypercortisolism. This study shows that #31 is an effective inhibitor of cortisol production and SF-1 target gene expression *in vitro*. Its effects *in vivo* and its effects on ACT cells are worthwhile to investigate further. In conclusion, inhibition of SF-1 activity shows much promise as a potential future treatment option in canine hypercortisolism.

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Part

III

Prognostic factors

Chapter

7

The Utrecht Score: A Novel Histopathological Scoring System to Assess the Prognosis of Dogs with Cortisol-Secreting Adrenocortical Tumors

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Abstract

A cortisol-secreting adrenocortical tumor (ACT) is the cause of naturally occurring canine hypercortisolism in approximately 15-20% of cases. The differentiation between an adrenocortical adenoma and carcinoma is usually based on histopathology. However, histopathological parameters have never been linked to the dogs' survival. Moreover, in human medicine the interobserver variability of some histopathological parameters that are used for ACTs is high. The objective of this study was to establish a reliable and easy-to-use histopathological scoring system for cortisol-secreting ACTs that can assess the prognosis of dogs after adrenalectomy. Cortisol-secreting ACTs of 50 dogs, collected between 2002 and 2015, were included in this study. Twenty histopathological features were assessed by one veterinary pathologist and one resident in veterinary pathology. In addition, the Ki67 proliferation index was assessed by two observers. Only parameters with intra- and interobserver agreement scores (intraclass correlation or Cohen's kappa coefficient) of ≥ 0.40 were included in survival analyses. Use of multivariate forward stepwise regression analysis with associated hazard ratios led us to a scoring system which we call the Utrecht score: the Ki67 proliferation index, + 4 if more than 33% of the tumor cells have clear/vacuolated cytoplasm, and + 3 if necrosis is present. Using cut-off values of 6 and 11, we could distinguish three groups that had significantly shorter survival times with increasing Utrecht scores. We conclude that the Utrecht score can be used to assess the prognosis of dogs with cortisol-secreting ACTs after adrenalectomy, which can help to select high-risk dogs that might benefit from adjuvant treatment or additional monitoring.

Introduction

Naturally occurring hypercortisolism is one of the most common endocrine disorders in dogs. It is caused by a cortisol-secreting adrenocortical tumor (ACT) in approximately 15 to 20% of cases, for which the treatment of choice is adrenalectomy, if no metastases can be detected.[1]

Without the presence of metastases, assessing the malignancy of an ACT remains challenging. The differentiation between an adrenocortical adenoma (ACA) and adrenocortical carcinoma (ACC) is usually based on histopathology. Although several studies have

been conducted on the histopathological analysis of human ACTs,[2–9] the literature on canine ACTs is less extensive. The most recent study on histopathology of canine ACTs was published in 2004, in which the authors compared a number of histopathological criteria between ACAs and ACCs.[10] The criteria that were determined to be diagnostically useful included tumor size, peripheral fibrosis, capsular invasion, trabecular growth pattern, hemorrhage, necrosis, single-cell necrosis, hematopoiesis, fibrin thrombi, cytoplasmic vacuolation and the proliferation marker Ki67.[10] However, whether the presence or absence of these parameters was associated with poor survival times has not been assessed. Moreover, the parameters used in this study were analyzed by only one pathologist,[10] whereas in the classification of human ACTs the interobserver variability is known to be high for some of these parameters.[2,3]

In humans, the most widely used histopathological scoring system to differentiate ACCs from ACAs is the Weiss score. In this system, the presence of three or more out of nine established histopathologic criteria indicates malignant potential.[6,7] Although the Weiss score can diagnose an ACC with high sensitivity and specificity, some of the criteria included suffer from interobserver variation and require evaluation by an experienced endocrine pathologist.[2,3] To reduce this interobserver variation, other scoring systems such as the Weiss revisited score[3] and the Helsinki score[2] have been proposed. In addition, several studies have shown the value of immunohistochemical staining for the proliferation marker Ki67 to differentiate between ACAs and ACCs.[3,11–14]

In humans, the prognosis of patients with an ACC does not only differ from that of patients with an ACA, but the prognosis also varies greatly within the group of patients with an ACC.[8,15] In human ACCs, histopathological criteria that are associated with a poor prognosis include high mitotic rate, high Ki67 proliferation index (PI), and high Helsinki score.[7,8,16,17]

The objective of this retrospective study was to establish a reliable and easy-to-use histopathological scoring system for cortisol-secreting ACTs that can assess the prognosis of dogs after adrenalectomy.

Materials and methods

Case selection

Canine cortisol-secreting ACTs were collected between 2002 and 2015. Permission to use the ACT tissue was obtained from all dog owners. The suspicion of hypercortisolism was based on the presence of clinical signs and routine laboratory findings consistent with hypercortisolism. Non-suppressible hypercortisolism was diagnosed using the low-dose dexamethasone suppression test, or urinary corticoid:creatinine ratios (UCCRs) combined with a high-dose dexamethasone suppression test.[18] The presence of an adrenal

tumor was visualized by abdominal ultrasonography, computed tomography, or both. All dogs underwent unilateral adrenalectomy, which was performed by one of four experienced veterinary surgeons. Dogs were excluded from the study when they were euthanized or died before, during, or within two weeks after adrenalectomy; when the dog had bilateral adrenal tumors; when metastases were detected before or at time of surgery; when no formalin-fixed paraffin-embedded tissue was available; and when less than three months of follow-up information was available.

Clinical parameters

The dogs' medical records were retrospectively reviewed for dog-related parameters, clinical tumor-related parameters, and surgery-related parameters. Dog-related parameters included sex and neutering status, body weight, age at time of surgery, and preoperative treatment for hypercortisolism. Tumor-related parameters included the location of the tumor (left or right), whether there was evidence of venous invasion seen during diagnostic imaging or during surgery, and the tumor diameter (not including normal adrenal tissue). Measurement of the tumor diameter was performed during diagnostic imaging (ultrasound or CT) and/or after surgery. Surgery-related parameters included the duration of the surgery, and whether the tumor capsule ruptured during surgery.

Histopathological parameters

The ACTs were collected within 10 minutes after surgical removal. A representative section of the tumor was fixed in formaldehyde for histopathology and immunohistochemistry, the remaining tumor sections were used for other research purposes. The tissues were fixed in 4% buffered formaldehyde for 24 to 48 hours, embedded in paraffin, and cut into 4-µm sections. One tissue section for each ACT was stained with hematoxylin and eosin, and one with the Gordon and Sweet's reticulin stain. Twenty histopathological parameters were assessed by one veterinary pathologist (GG) and one resident in veterinary pathology (KC): 1) reticulin fiber density; 2) growth pattern; 3) morphology of cytoplasm; the presence or absence of 4) hemorrhage, 5) extramedullary hematopoiesis, 6) intratumoral fibrosis, 7) peripheral fibrosis, 8) capsular invasion, 9) venous invasion, 10) sinusoidal invasion, 11) fibrin thrombi, 12) necrosis, 13) abnormal mitotic figures, 14) nucleoli, and 15) nuclear chromasia; and establishment of the 16) number of cells undergoing single cell necrosis, 17) number of mitotic figures, 18) nuclear size, 19) cellular size, and 20) nuclear grade.

The reticulin fiber density was scored as the percentage of the ACT with reduced fiber density compared to the density of reticulin fibers in the zona fasciculata of a normal adrenal gland. The growth patterns were evaluated as being diffuse, nesting, or trabecular, and the growth pattern that predominated was recorded. The reticulin fiber density and the growth patterns were evaluated in the Gordon and Sweet's reticulin stain, all other parameters in the hematoxylin and eosin stain. An estimate of the percentage of cells with clear/

vacuolated cytoplasm was noted. Peripheral fibrosis was considered to be present when a multi-layered band of fibrous tissue surrounded at least part of the ACT. Capsular invasion was considered to be present when the ACT infiltrated or perforated the peripheral capsule; small nests of adrenocortical cells that resembled cells of the zona glomerulosa within the capsule were considered to be normal, because these nodules are also often encountered in adrenal glands of healthy dogs. Necrosis was considered to be present when confluent nests of necrotic cells were visible. For the number of cells undergoing single-cell necrosis and the number of mitotic figures, first the sections were screened to determine where most cells undergoing single-cell necrosis or mitosis appeared to be present, and then these parameters were evaluated in the one high power field (HPF; 400x magnification) where they seemed to be most abundant. Nuclear and cellular sizes were evaluated and given a number relative to a nucleus or cell in the zona fasciculata of a normal adrenal gland (e.g., equally large: 1; twice as large: 2), the size that predominated was recorded. The nuclear grade was evaluated as described by Fuhrman *et al.* (1982).[19] All histopathological parameters were visually estimated in the entire tissue section using a light microscope, no image analysis was performed. Both observers assessed the tissue sections twice at separate time points, and were blinded to clinical data.

Ki67 proliferation index

For Ki67 immunohistochemistry, one tissue section per ACT was rehydrated in a series of xylene and ethanol baths. Antigen retrieval was performed with Tris-EDTA buffer (pH 9) in a microwave, at 850W for 7 minutes and at 450W for 15 minutes. After the slides were cooled down they were incubated with 0.35% H202 in Tris buffered saline (TBS) for 30 minutes to block endogenous peroxidase. The slides were blocked with 10% normal goat serum with 1% bovine serum albumin (BSA) in TBS for 30 minutes. Incubation with a mouse monoclonal primary anti-Ki67 antibody (MIB-1 clone, M7240, Dako, Agilent, Amstelveen, The Netherlands), diluted 1:75 in 1% BSA in TBS, took place overnight at 4°C. The following day the slides were incubated with secondary antibody (HRP-labelled goat-anti-mouse, EnVision+, Dako) for 30 minutes. The slides were incubated with Dako Liquid DAB+ Substrate Chromogen System (K3468, Dako) for 10 minutes, and counterstained with hematoxylin. A series of ethanol and xylene baths were used to dehydrate the slides, after which the slides were mounted with VectaMount™ Mounting Medium (H-5000, Vector Laboratories, Peterborough, United Kingdom). During intermediate steps the slides were washed in TBS. Canine colon tissue slides were used as positive control tissue, and the primary antibody was replaced by normal mouse IgG (SC3877, Santa Cruz Biotechnology, Heidelberg, Germany) for the negative control.

The Ki67 PI was assessed by the resident in veterinary pathology (KC) and a researcher with experience in adrenocortical tumors (KS) in hot spot areas, which were the areas that appeared to have the highest percentage of Ki67 positive cells. For each tissue slide five images were captured on an Olympus BX60 microscope with Leica LAS-AF software, at 200x magnification. A minimum of 1000 nuclei were counted in total per ACT on the one

or two images with the highest amount of Ki67 positive cells, for which ImageJ software[20] was used to keep track of the number of counted positive and negative cells. Only nuclear staining was considered to be positive. Care was taken to only include ACT cells and not, e.g., cells of the stromal compartment or extramedullary hematopoiesis. The PI was calculated as the percentage of Ki67 positive nuclei relative to the total number of counted nuclei for each ACT.

Analyses

The intra- and interobserver agreement scores for histopathological parameters and the Ki67 PI were quantified using the intraclass correlation coefficient (ICCC) for continuous variables, and Cohen's kappa coefficient for categorical variables. The strength of agreement was interpreted as follows: <0.40, poor; 0.40-0.59, moderate; 0.60-0.79, good; 0.80-1.00, excellent.[21] Only parameters with an agreement score of more than 0.40 in all areas (i.e. intraobserver agreement of both observers, and interobserver agreement) were included in survival analyses. In case of continuous variables, the results of all four observations (i.e. two observations per observer) were averaged for further analyses. In case of categorical variables, the result that was most prevalent was noted. If this could not be established, the parameter was scored again for these slides and the final outcome was noted.

Dogs were considered to have died as a result of the ACT when they were euthanized because of metastases or comorbidities related to recurrence of hypercortisolism. Recurrence of hypercortisolism was confirmed by the presence of hypercortisolism-related clinical signs and elevated UCCRs, which could be either due to regrowth of the ACT or metastases. For each dog, survival time was calculated from the time of surgery to euthanasia due to recurrence. If a dog died from unrelated causes, was lost to follow-up, or was still alive at the end of the study, then the dog was censored and the last known date that the dog was still alive was used as censoring date.

Univariate analyses were performed with the Cox proportional hazards model. All variables that had a P-value of < 0.15 in the univariate analyses were subsequently included in multivariate stepwise regression with forward selection. Optimal cut-off values were calculated with receiver operating characteristic curves. The value with the highest Youden index (sensitivity + specificity – 1) was selected as the optimal cut-off value. Survival times were calculated using the Kaplan–Meier product-limit method. The log-rank method was used to calculate if differences between groups were significant.

P-values of < 0.05 were considered significant. All statistical analyses were performed with SPSS Statistics for Windows (Version 24.0, IBM Corp, Armonk, NY).

Table 1 Effects of clinical parameters on survival: univariate analyses

Parameter	Distribution	Total	Hazard ratio (95% CI)	P-value
Sex	25 male, 25 female	50	1.046 (0.420-2.607)	0.921
Neutered	22 no, 28 yes	50	1.483 (0.574-3.814)	0.414
Body weight	14.2 (3.6-74.7) kg	50	1.011 (0.979-1.045)	0.506
Age	10.0 (2.1-12.9) yr	50	1.099 (0.844-1.431)	0.483
Treated before surgery	36 no, 9 yes	45	1.060 (0.346-3.250)	0.918
Location	23 left, 25 right	48	1.263 (0.502-3.176)	0.620
Venous invasion (macro)	27 no, 16 yes	43	0.722 (0.277-1.882)	0.505
Tumor diameter	2.5 (1.0-10.0) cm	46	1.424 (1.110-1.827)	*0.005**
Surgery duration	156 (42-290) min	43	1.002 (0.993-1.012)	0.630
Capsule rupture	31 no, 16 yes	47	1.339 (0.525-3.415)	0.541

Univariate analyses performed with the Cox proportional hazards model. Distribution is indicated in categories for categorical variables, and in median (with the range in parentheses) for continuous variables. Total indicates the total number of dogs for which this parameter was known. Hazard ratio (with the 95% confidence interval in parentheses) indicates the hazard of the second category compared to the first in case of categorical parameters, and the hazard per stated unit in case of continuous variables. Significant P-values are indicated in italic with an asterisk.

Results

Cases

A total of 50 dogs were included in the study. The most represented dog breeds were Labrador Retriever (5), Dachshund (5), Jack Russel Terrier (4), Schnauzer (2), Maltese (2), Fox Terrier (2), and White Shepherd (2). Of the remaining dogs, 11 were mixed-breed dogs and 17 were of breeds that were represented once. Information on additional dog-related parameters is included in Table 1.

The estimated median survival time after adrenalectomy for all 50 dogs as calculated by the Kaplan-Meier method was 54.7 months (95% CI 47.1 – 62.2 months) (Fig. 1A). Of the 50 dogs, 19 were known to have had recurrence of hypercortisolism. The median survival time of these 19 dogs with recurrence was 16.9 months (95% CI 10.8 – 49.3 months). All 31 dogs that had no recorded recurrence were censored in the survival analyses so no estimated median survival was reached using the Kaplan-Meier method, but the median follow-up time was 27.1 months (95% CI 15.4 – 42.0 months).

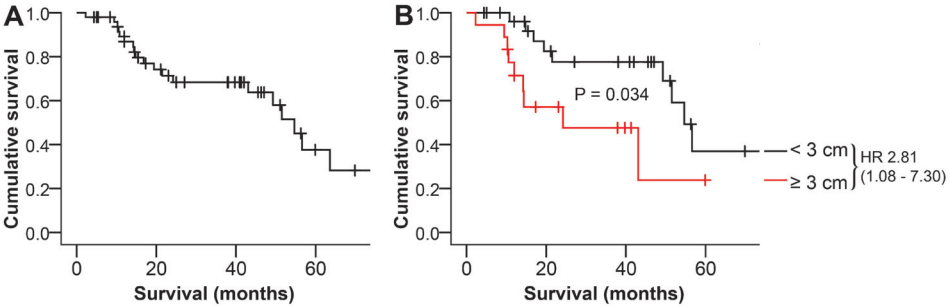

Figure 1 (A) Overall survival of all dogs included in the study (n = 50) and (B) survival stratified according to tumor diameter (n = 46) using Kaplan-Meier analysis. Survival times were calculated from the time of surgery to euthanasia due to recurrence. Censored dogs are indicated as tick marks. (B) Dogs were classified as having an adrenocortical tumor diameter < 3 cm (black line, n = 28), or ≥ 3 cm (red line, n = 18). HR indicates estimated hazard ratio (with 95% confidence interval designated in parentheses). P-value indicates the significance of the difference between the groups as calculated with the log-rank test.

Clinical parameters

Of all clinical parameters that were analyzed, only the tumor diameter was significantly associated with survival (univariate analysis, Table 1). The optimal cut-off value was approximately 3 cm, which resulted in a significant (P = 0.034) difference in survival times between dogs with an ACT ≥ 3 cm (n = 18, estimated median survival time 24.2 months, 95% CI 1.3 – 47.1 months) and dogs with an ACT < 3 cm (n = 28, estimated median survival time 54.7 months, 95% CI 48.2 – 61.1 months) (Fig. 1B). Cut-off values of 2 cm or 5 cm as previously suggested in literature[10,22] did not result in significant differences in survival times (P = 0.255 and P = 0.172, respectively).

Histopathological parameters and Ki67 proliferation index

In assessing intra- and interobserver reliability, four histopathological parameters had agreement scores of more than 0.40 in all areas (i.e. intraobserver agreement scores for both observers, and interobserver agreement score): decreased reticulin fiber density (Figure 2A, lowest agreement score 0.56, P < 0.001), hematopoiesis (Figure 2B, lowest agreement score 0.59, P < 0.001), clear/vacuolated cytoplasm (Figures 2C and 2D, lowest agreement score 0.67, P < 0.001), and necrosis (Figure 2E, lowest agreement score 0.51, P < 0.001). No intraobserver agreement scores could be calculated for the Ki67 PI (Figure 2F) because both observers assessed all ACTs once, but the interobserver agreement score was excellent (0.96, P < 0.001). All intra- and interobserver agreement scores are shown in Supplemental Table 1.

In assessing the potential of the histopathological parameters as prognostic indicators, three parameters were significantly associated with survival according to univariate analyses (Table 2): the percentage of clear/vacuolated cytoplasm, the presence of necrosis, and the Ki67 PI. For the continuous variables that were significantly associated with survival,

Table 2 Effects of histopathological parameters and the Ki67 PI on survival: univariate analyses

Parameter	Distribution	Total	Hazard ratio (95% CI)	P-value
Decreased reticulin fibre density	85 (19-100) %	50	1.018 (0.987-1.051)	0.257
Clear/vacuolated cytoplasm	48 (5-95) %	50	1.023 (1.004-1.043)	*0.020**
Hematopoiesis	37 no, 13 yes	50	0.882 (0.284-2.737)	0.828
Necrosis	30 no, 20 yes	50	3.495 (1.336-9.141)	*0.011**
Ki67 proliferation index	3 (0-22) %	50	1.174 (1.065-1.295)	*0.001**

Univariate analyses performed with the Cox proportional hazards model. Distribution is indicated in categories for categorical variables, and in median (with the range in parentheses) for continuous variables. Total indicates the total number of dogs for which this parameter was known. Hazard ratio (with the 95% confidence interval in parentheses) indicates the hazard of the second category compared to the first in case of categorical parameters, and the hazard per stated unit in case of continuous variables. Significant P-values are indicated in italic with an asterisk.

we calculated optimal cut-off values, for which dogs that had recurrence and died within 30 months after surgery were included in the positive group (n = 13), and dogs that had no recorded recurrence and lived for at least 30 months were included in the negative group (n = 15). For the percentage of clear/vacuolated cytoplasm, the optimal cut-off value was approximately 33%. For the Ki67 PI, the optimal cut-off value was 4.6%.

The Utrecht score

To determine which parameters were independent predictors of poor survival, we used multivariate logistic regression with forward selection. This indicated that the Ki67 PI, clear/vacuolated cytoplasm in at least 33% of the tumor cells, and the presence of necrosis were all independent predictors of survival. Based on their hazard ratios in the multivariate analysis (Table 3), this led to the scoring system: 1.12 × Ki67 PI, + 4.35 if clear/vacuolated cytoplasm was present in at least 33% of the tumor cells, + 3.18 if necrosis was present. We simplified this system to: the Ki67 PI, + 4 if ≥ 33% of tumor cells have clear/vacuolated cytoplasm, + 3 if necrosis is present, which we call the Utrecht score (Fig. 3).

The median Utrecht score was 7.1 (range, 0.4 – 29.2), and the optimal cut-off value was approximately 6. To further divide the ACTs with scores of 6 or higher into two groups with different survival times, we used a cut-off value of 11, which was calculated as the approximate optimal cut-off value in the subset of patients with scores of more than 6. We then categorized the ACTs according to their Utrecht score in three groups: (1) Utrecht score < 6 (n = 17), (2) Utrecht score ≥ 6 to < 11 (n = 22), and (3) Utrecht score ≥ 11 (n = 11). As illustrated in Figure 4, the estimated median survival time was not reached for group 1, was 51.5 months (95% CI 43.7 – 59.3) for group 2 (significantly different from group 1, P = 0.012), and 14.4 months (95% CI 10.5 – 18.2) for group 3 (significantly different from group 2, P = 0.005).

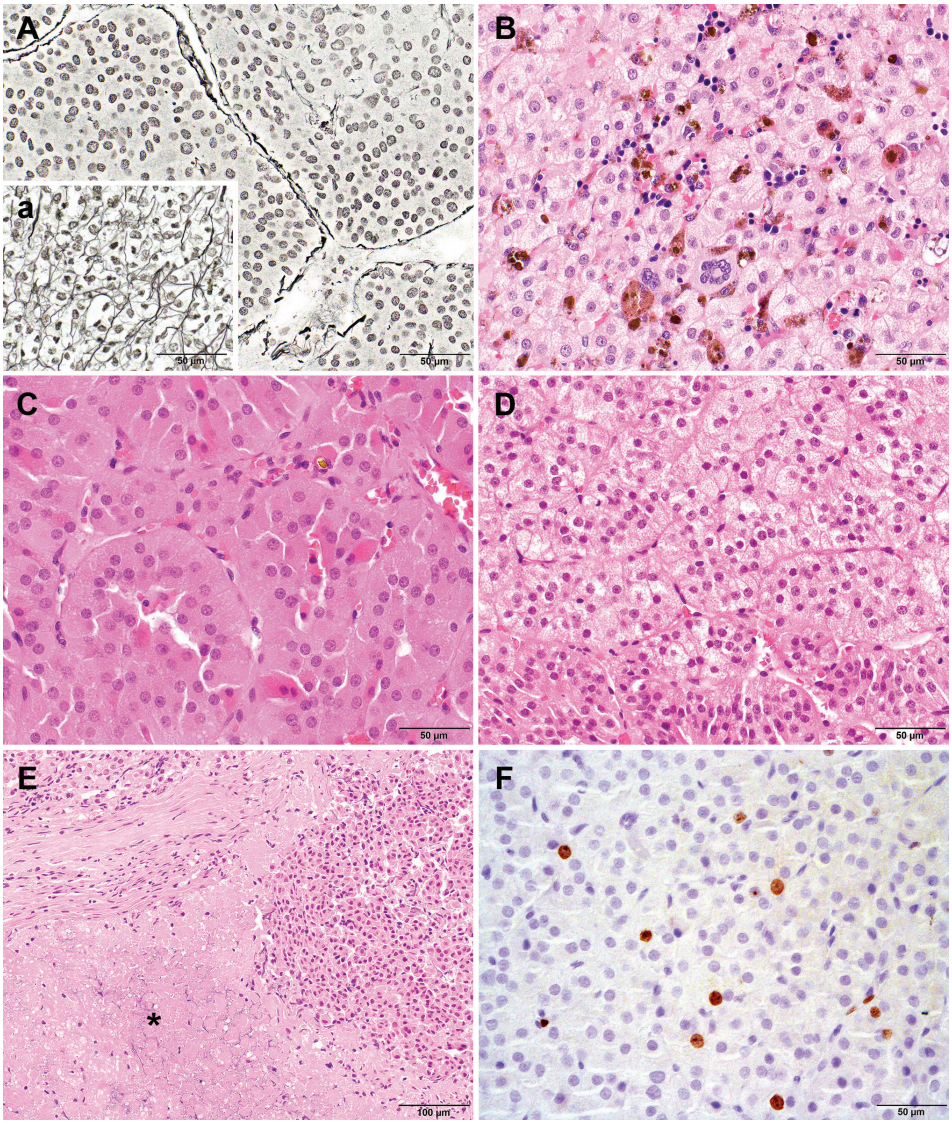

Figure 2 Histopathological parameters in canine cortisol-secreting adrenocortical tumors with intra- and inter-observer agreement scores of > 0.4. (A) Example of tumor area with decreased reticulin fiber density compared to (a) the zona fasciculata of a normal adrenal gland. Gordon and Sweet's reticulin stain, bar represents 50 µm. (B) Example of tumor with extramedullary hematopoiesis, including megakaryocytes. Hematoxylin and eosin stain, bar represents 50 µm. (C) Example of adrenocortical tumor cells with eosinophilic/granular cytoplasm. Although some tumor cells do contain cytoplasm with a small number of vacuoles, the cytoplasm of the cells in this image was generally characterized as eosinophilic/granular. Hematoxylin and eosin stain, bar represents 50 µm. (D) Example of adrenocortical tumor cells with clear/vacuolated cytoplasm. The majority of cells in this image are characterized by cytoplasm with numerous small vacuoles. The cells in the bottom part of the image contain cytoplasm with a more eosinophilic/granular aspect. Hematoxylin and eosin stain, bar represents 50 µm. (E) Example of an adrenocortical tumor in which part of the cells are necrotic (asterisk). Hematoxylin and eosin stain, bar represents 100 µm. (F) Example of an adrenocortical tumor with Ki67 positive staining (brown) within the nuclei of a subset of tumor cells. Anti-Ki67 immunohistochemical staining with hematoxylin counterstaining, bar represents 50 µm.

Table 3 Independent predictors of survival: multivariate analysis

Parameter	Distribution	Hazard ratio (95% CI)	P-value
Ki67 proliferation index	3.1 (0.0-22.2) %	1.124 (1.018-1.242)	*0.021**
Clear/vacuolated cytoplasm in ≥ 33%	18 no, 32 yes	4.350 (1.224-15.455)	*0.023**
Presence of necrosis	30 no, 20 yes	3.181 (1.103-9.173)	*0.032**

Multivariate stepwise regression performed with the Cox proportional hazards model with forward selection. Distribution is indicated in categories for categorical variables, and in median (with the range in parentheses) for continuous variables. Hazard ratio (with the 95% confidence interval in parentheses) indicates the hazard of the second category compared to the first in case of categorical parameters, and the hazard per stated unit in case of continuous variables. Significant P-values are indicated in italic with an asterisk.

Discussion

Here we introduce the Utrecht score: a novel histopathological scoring system that can assess the prognosis of dogs with a cortisol-secreting ACT after adrenalectomy. With cut-off values of 6 and 11, we could distinguish three groups that had significantly shorter survival times with increasing scores. We propose to classify ACTs with a score of < 6 as having low risk of recurrence, with a score of ≥ 6 to < 11 as having moderate risk of recurrence, and with a score of ≥ 11 as having high risk of recurrence.

Because most veterinary pathologists won't encounter an ACT on a daily basis, histopathological assessment of an ACT ideally should not require extensive training. In this study, 20 histopathological parameters were scored twice by both an experienced veterinary pathologist and a resident in veterinary pathology. This allowed us to include only those parameters that turned out to have low intra- and interobserver variability irrespective of the observers' experience, thereby improving the reliability of the Utrecht score.

A recent study on human ACCs reported low intra- and interobserver agreement scores for Ki67 scoring among 14 trained endocrine pathologists.[23] However, in that study each observer was allowed to perform the analyses according to their own method of preference, which included visual estimation, formal manual count, and digital image analysis. In our study, the interobserver agreement of the Ki67 PI was excellent, which is most likely associated with the methodology used. To standardize Ki67 PI scoring as much as possible, we suggest to analyze the Ki67 PI in hot spot areas, to capture images of these areas, to count at least 1000 nuclei, and to use ImageJ[20] or similar software to keep track of the number of counted positive and negative cells. A drawback of this method is that it is time-consuming, but developments in digital microscopy-enabled methods could facilitate future reproducible and reliable Ki67 PI assessments.[23]

Increased cell proliferation and tumor hypoxia are both important features of aggressive cancers,[24] which explains why Ki67 PI and necrosis are important prognostic factors in canine ACTs. Why the high percentage of clear/vacuolated cytoplasm is an important factor is less clear. The appearance of clear/vacuolated cytoplasm likely indicates the presence of intracytoplasmic lipid droplets, which are extracted during histologic specimen preparation unless special methods are used.[25] Since cholesterol, which could be stored in these lipid droplets, functions as substrate for all steroid hormones, the percentage of cells with clear/vacuolated cytoplasm could be related to the production of cortisol or its precursors. In human ACTs, cortisol production is known to be a negative prognostic indicator.[26,27] In our study, however, only cortisol-secreting ACTs were included so the presence or absence of hypercortisolism was not a variable, but the percentage of cells with clear/vacuolated cytoplasm could have been related to the degree of hypercortisolism. Because different methods or assays were used to establish hypercortisolism, we were unable to test this hypothesis. For future studies it would be interesting to determine whether clear/vacuolated cytoplasm is indeed related to the degree of cortisol production, since this would also indicate that non-secreting ACTs might be less malignant overall than cortisol-producing ACTs.

Of the clinical parameters, only the tumor diameter was significantly associated with survival. However, it did not retain its significance in the multivariate analysis, neither on a continuous scale nor when using the indicated cut-off value. Although this means that it is not an independent predictor of survival after surgery, it is currently the only parameter that can give an assessment of prognosis before surgery. We showed that dogs with a tumor diameter of ≥ 3 cm had significantly worse survival times after adrenalectomy than those with a diameter of < 3 cm. Although previous studies reported that cut-off values of 2 cm[10] or 5 cm[22] should be used, classification based on these values did not result in significantly different survival times in our study. In some studies tumor size was not significantly associated with survival time.[28,29] For future studies it might be useful to determine whether measurement of the tumor volume has additional prognostic value compared to measurement of the tumor diameter.

In this study we assessed whether the evaluated parameters can predict long-term survival. What we therefore did not assess was whether these parameters influence perioperative or short-term survival. For example, although venous invasion as observed during imaging or surgery does not seem to affect long-term survival, as also reported in other studies,[30,31] it could complicate the surgical procedure. Indeed, extensive invasion of the tumor into the caudal vena cava when the tumor thrombus extends beyond the hepatic hilus has been reported to increase perioperative mortality.[31] In other studies, however, the presence of venous invasion did not affect perioperative mortality,[10,30] so this possibly depends on the extent of invasion, the experience of the surgeon, and/or whether cases with extensive venous invasion are considered to be candidates for surgery. Other factors that have been reported to increase perioperative mortality rates are acute adrenal hemorrhage and large tumors.[30]

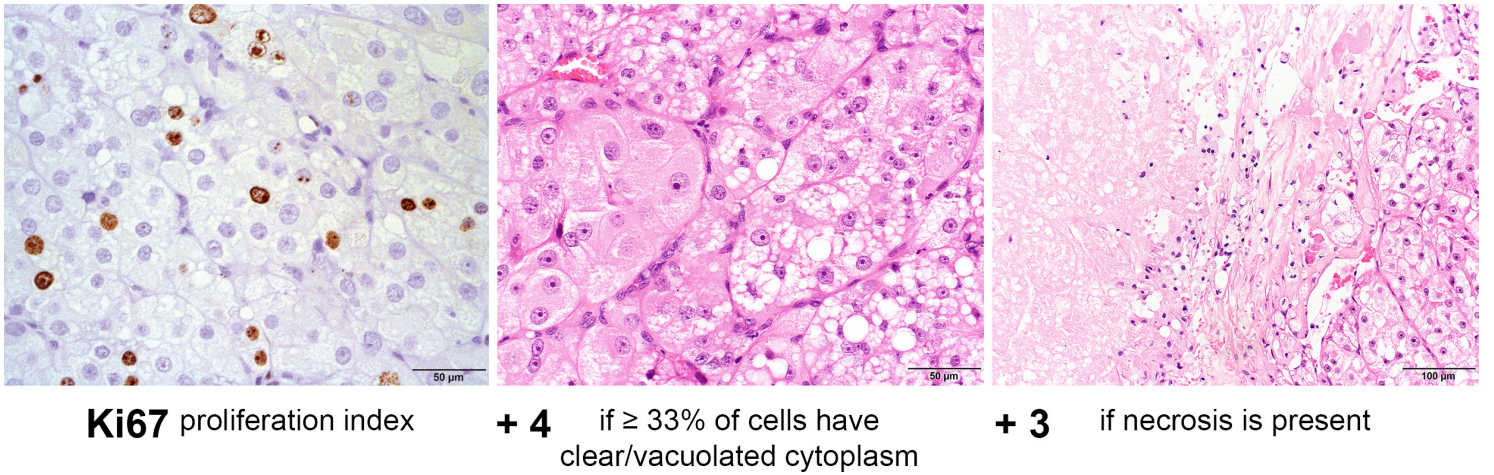

Figure 3 The Utrecht score in canine adrenocortical tumors: Ki67 proliferation index, + 4 if ≥ 33% of tumor cells have clear/vacuolated cytoplasm, + 3 if necrosis is present. The images of the parameters shown here are from the adrenocortical tumor of one patient, which had the highest Utrecht score of our dataset. The adrenocortical tumor in this example had a Ki67 proliferation index of 22.2%, clear/vacuolated cytoplasm (small to very large vacuoles) in approximately 66% of tumor cells (so more than 33%), and fields of necrosis were present. This resulted in a total score of 22.2 + 4 + 3 = 29.2.

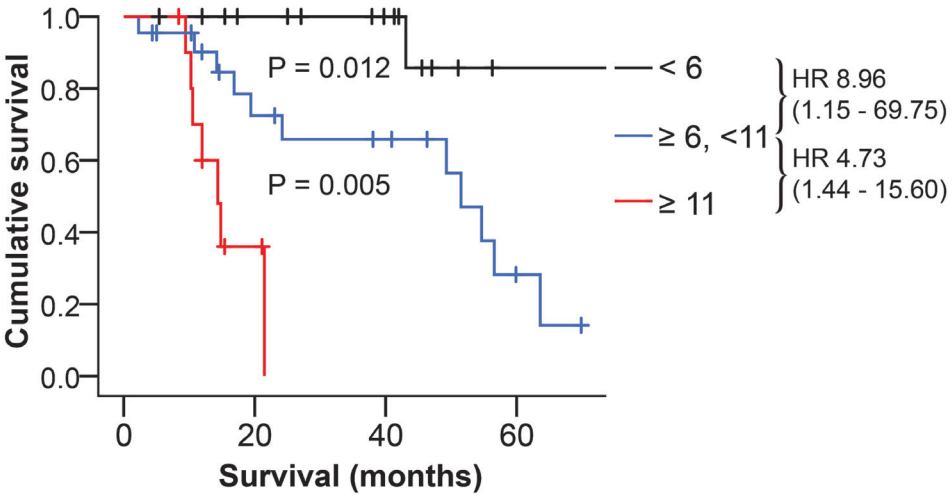

Figure 4 Survival stratified according to the Utrecht score using Kaplan-Meier analysis. Survival times were calculated from the time of surgery to euthanasia due to recurrence. Censored dogs are indicated as tick marks. Dogs were classified as having a Utrecht score of < 6 (black line, n = 17), of ≥ 6 to < 11 (blue line, n = 22), or of ≥ 11 (red line, n = 11). HR indicates estimated hazard ratios (with 95% confidence interval designated in parentheses). P-values indicate the significance of the difference between the groups as calculated with the log-rank test.

When comparing the Utrecht score with scoring systems for human ACTs, it is noteworthy that it closely resembles the Helsinki score (3 × mitotic rate + 5 × necrosis + Ki67 PI).[2] Because the intra- and interobserver agreement scores for the assessment of mitotic figures were inadequate in our study, we did not include the mitotic rate in our survival analyses. The agreement scores for the mitotic rate could possibly be improved by counting the number of mitotic figures in more HPFs. Interestingly, in both the Weiss and Weiss revisited score, clear cytoplasm in less than 25% of the tumor is a negative prognostic factor,[3,6] similar to previously described in canine ACTs,[10] whereas in the Utrecht score more than 33% tumor cells with clear/vacuolated cytoplasm is a negative prognostic factor. Clear cytoplasm was, however, reported to be the least useful criterion in the Weiss score.[6] In both the human and canine previous studies, cases with an ACT were selected irrespective of their hormonal status,[3,6,10] whereas we included only cortisol-secreting ACTs. Whether this discrepancy in morphology of cytoplasm is related to a difference in assessment, interpretation, or both, or to differences in the hormonal status of the ACT, remains to be elucidated.

A weakness of this study is its retrospective nature. Not all information had been documented consistently, e.g. information on the tumor diameter was missing in four cases, and measurement of the tumor diameter was not standardised. Moreover, we were not able to reliably calculate tumor volumes because the multiple dimensions of the tumor were not often documented, nor was the tumor weight, which has prognostic value in human ACTs.[9] The study's retrospective nature could also have affected the reported survival times of the dogs, which were sometimes based on the owner's estimate of when their dog

had died. In addition, the reason for recurrence was not investigated in every case, so we could not make a distinction between local recurrence and recurrence due to metastases.

Another remark is that although ACTs can be highly heterogeneous, all histopathological parameters and the Ki67 PI have been assessed on just one tissue section of the ACT due to the retrospective nature of this study. If the most malignant area of the tumor was not included in this tissue section, this could have resulted in an underestimation of the malignancy grade. However, even with just one tissue section the Utrecht score was able to distinguish three groups with significantly different survival times.

Although the fact that we only included parameters with intra- and interobserver agreement scores of ≥ 0.4 improves the reliability of the scoring system, a drawback is that we may have excluded factors that are important prognostic parameters. Moreover, the method used to create the Utrecht score has based its calculations on this specific subset of patients. To verify the validity of the score, future studies in other patient groups and with the participation of more veterinary pathologists are required. Additionally, in this study we only included cortisol-secreting ACTs, and whether the score can also be a valuable prognostic tool in, e.g., non-secreting ACTs will have to be determined in future studies.

In conclusion, we introduce the Utrecht score to post-surgically assess the prognosis of dogs with cortisol-secreting ACTs. Having an accurate assessment of prognosis is useful to communicate to the dog's owner, but could also assist in selecting high-risk dogs that might benefit from additional monitoring, adjuvant therapy with, e.g., mitotane, or both. Moreover, a solid histopathological scoring system represents a basis for future studies on other markers of malignancy, which could facilitate the identification of potential future treatment targets.

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Conflict of Interest Declaration: The authors declare no conflict of interest.

Supplemental Table 1 Intra- and interobserver agreement scores

Parameter	Intraobserver 1	Intraobserver 2	Interobserver
Reticulin fiber density %	0.641 [a] P < 0.001	0.906 [a] P < 0.001	0.557 [a] P < 0.001
Growth pattern	0.150 [b] P = 0.071	0.266 [b] P = 0.007	0.047 [b] P = 0.613
Clear/vacuolated cytoplasm %	0.861 [a] P < 0.001	0.796 [a] P < 0.001	0.665 [a] P < 0.001
Hemorrhage	0.729 [b] P < 0.001	0.606 [b] P < 0.001	0.397 [b] P < 0.001
Hematopoiesis	0.748 [b] P < 0.001	0.883 [b] P < 0.001	0.587 [b] P < 0.001
Intratumoral fibrosis	0.168 [b] P = 0.118	0.348 [b] P = 0.001	0.159 [b] P = 0.096
Peripheral fibrosis	0.269 [b] P = 0.002	N/A	N/A
Capsular invasion	0.327 [b] P = 0.006	N/A	0.097 [b] P = 0.381
Venous invasion	0.925 [b] P < 0.001	0.389 [b] P < 0.001	0.351 [b] P = 0.004
Sinusoidal invasion	-0.048 [b] P = 0.647	-0.049 [b] P = 0.673	-0.066 [b] P = 0.572
Fibrin thrombi	0.516 [b] P < 0.001	0.784 [b] P < 0.001	0.283 [b] P = 0.016
Necrosis	0.619 [b] P < 0.001	0.670 [b] P < 0.001	0.511 [b] P < 0.001
Abnormal mitotic figures	N/A	N/A	N/A
Nucleoli	0.462 [b] P < 0.001	-0.060 [b] P = 0.568	0.305 [b] P = 0.009
Nuclear chromasia	-0.027 [b] P = 0.767	N/A	N/A
Single cell necrosis	0.042 [b] 0.733	-0.260 [b] P = 0.688	-0.122 [b] P = 0.307
Mitotic figures	0.622 [a] P < 0.001	-0.028 [a] P = 0.546	0.484 [a] P = 0.005
Nuclear size	0.630 [b] P < 0.001	0.479 [b] P < 0.001	0.319 [b] P = 0.019
Cellular size	0.292 [b] P = 0.082	0.233 [b] P = 0.038	0.286 [b] P = 0.090
Nuclear grade	0.238 [a] P < 0.001	0.605 [a] P < 0.001	0.321 [a] P = 0.008
Ki67 proliferation index	N/A	N/A	0.959 [a] P < 0.001

Intra- and interobserver agreement scores of histopathological parameters in canine cortisol-secreting adrenocortical tumours (n = 50). Method of score quantification indicated by [a] (intraclass correlation coefficient; continuous variables) and [b] (Cohen's kappa coefficient; categorical variables). N/A: not available; indicates that at least one of the variables tested is a constant value.

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Chapter

8

Molecular Markers of Malignancy in Canine Cortisol-Secreting Adrenocortical Tumors

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Submitted

Abstract

Background: Hypercortisolism is caused by a cortisol-secreting adrenocortical tumor (ACT) in approximately 15-20% of cases in dogs. Little is known about which molecular markers are associated with malignant behavior of canine ACTs. *Objectives:* The objective of this study was to identify molecular markers of malignancy, which could be useful to refine prognostic prediction and to identify treatment targets. *Animals:* Cortisol-secreting ACTs were included from 40 dogs, of which follow-up information was available. The ACTs were classified as low risk of recurrence tumors (LRTs; n = 14) or moderate/high risk of recurrence tumors (MHRTs; n = 26), based on their histopathological Utrecht score. Normal adrenals (NAs) were included from 11 healthy dogs as reference material. *Methods:* The mRNA expression of 14 candidate genes was analyzed in the 40 ACTs and in 11 NAs with quantitative RT-PCR. The genes' expression levels were statistically compared between NAs, LRTs and MHRTs. Univariate and multivariate analyses were performed to determine the association of the genes' expression levels with survival. *Results:* Seven genes were differentially expressed between NAs and ACTs, of which pituitary tumor-transforming gene-1 (*PTTG1*) and topoisomerase II alpha (*TOP2A*) were also differentially expressed between LRTs and MHRTs. In survival analyses, high expression levels of Steroidogenic factor-1 (*SF-1*), *PTTG1* and *TOP2A* were significantly associated with poor survival. *Conclusions and clinical importance:* In this study we have identified several genes that are part of the molecular signature of malignancy in canine ACTs. These findings can be used to refine prognostic prediction, but also offer insights for future studies on druggable targets.

8

Introduction

Spontaneous hypercortisolism is one of the most common endocrine disorders in dogs, with an estimated prevalence of approximately 1 in 400.[1] It is caused by a cortisol-secreting adrenocortical tumor (ACT) in 15 to 20% of cases. If no metastases are detectable, the treatment of choice for an ACT is adrenalectomy. Other or adjuvant treatment options include the steroidogenesis inhibitor trilostane, which will only reduce the clinical signs of hypercortisolism and has no effect on tumor growth, and the adrenocorticolytic agent mitotane, which can cause considerable side-effects.[2]

Reported recurrence rates after adrenalectomy vary between 12 and 38%,[3–5] which can be caused by metastases or regrowth of the ACT. Assessing the risk of recurrence after adrenalectomy is usually based on histopathology. However, the histopathological parameters that are mostly used in the assessment of canine ACTs[6] often have high interobserver variability in human ACTs,[7,8] and some studies did not observe a significant difference in survival times of dogs after adrenalectomy based on their histopathological diagnosis.[4,9] To improve the reliability and prognostic value of histopathology in canine cortisol-secreting ACTs, we recently introduced a new histopathological scoring system: the Utrecht score.[5] The Utrecht score was based on parameters with low intra- and interobserver variability and their association with the dogs' survival times. It includes assessment of the Ki67 proliferation index, the presence of necrosis, and the percentage of clear/vacuolated cytoplasm, and increasing Utrecht scores were significantly associated with shorter survival times.[5]

In the most recent study on ACTs, the median survival time of 19 dogs with recurrence was 16.9 months (95% CI 10.8 – 49.3 months).[5] If these dogs would have been classified as having a high risk of recurrence, they could have received adjuvant treatment post-operatively which might have improved their survival times. Moreover, if molecular markers that are associated with malignancy and thus a high risk of recurrence could be identified, this could give more insight into which molecular pathways are useful to target for future treatment options. However, at present, little is known about which molecular markers are associated with malignancy of canine ACTs. Previous research by our group showed that the mRNA expression of Steroidogenic factor-1 (*SF-1; NR5A1*), an important regulator of adrenal development and steroidogenesis, was significantly higher in ACTs of dogs that had recurrence of hypercortisolism within 2.5 years after adrenalectomy, than in ACTs of dogs that had no recurrence for at least 2.5 years.[10] High expression of SF-1 is also an important negative prognostic indicator in human ACTs.[11,12] Other molecular markers that have prognostic value in human ACTs include pituitary tumor-transforming gene-1 (PTTG1),[13] topoisomerase II alpha (TOP2A),[14] Vav Guanine Nucleotide Exchange Factor 2 (VAV2),[15] and (for childhood ACTs) B-cell lymphoma 2 (BCL2).[16]

To identify molecular markers of malignancy in canine ACTs, we used a candidate gene approach. The genes we selected are involved in adrenal-specific pathways, proliferation/apoptosis-related pathways, or both, and were selected based on an estimation of their prognostic relevance and whether they or the pathways they are involved in are potential druggable targets. The results of this study could be used to refine prognostic classification and could provide more insight into which genes or pathways are interesting for future studies in new treatment options. To this end, we evaluated the mRNA expression of 14 candidate genes and assessed whether they were associated with the histopathological diagnosis of the ACT based on the Utrecht score, and/or with the dogs' survival times.

Materials and Methods

Case selection

Cortisol-secreting ACTs of dogs were collected between 2002 and 2015. All dog owners gave permission to use the ACT tissue for research purposes. The suspicion of hypercortisolism was based on clinical signs and routine laboratory findings that were consistent with hypercortisolism. A low-dose dexamethasone suppression test or urinary corticoid:creatinine ratios combined with a high-dose dexamethasone suppression test was used to diagnose non-suppressible (<50%) hypercortisolism.[17] The presence of an enlarged adrenal gland was visualized using abdominal ultrasonography or a computed tomography scan. All dogs underwent unilateral adrenalectomy, which was performed by one of in total of four experienced veterinary surgeons, all registered in The Netherlands as a veterinary specialist. Dogs were excluded from the study when they had bilateral adrenal tumors; when no formalin-fixed paraffin-embedded tissue was available for histopathological analysis; when no snap-frozen material was available for RNA isolation; when they were euthanized or died before, during, or within two weeks after adrenalectomy; and when less than three months of follow-up information was available. All dogs with ACTs in this study were also included in the previously published series where we introduced the Utrecht score.[5] The adrenal glands of 11 healthy dogs were used as reference material; these dogs were euthanized for reasons unrelated to the current study which was approved by the Ethical Committee of Utrecht University conform Dutch legislation.

Histopathological evaluation

The histopathological evaluation of each ACT was performed as described in the previously described series.[5] Formalin-fixed paraffin-embedded tissues were cut in 4 μm thick sections on Superfrost Plus™ Adhesion Microscope Slides (Thermo Fisher Scientific, Breda, The Netherlands). The tissue sections were stained with hematoxylin and eosin, and immunohistochemical staining for Ki67 (MIB-1 clone, M7240, Dako, Agilent, Amstelveen, The Netherlands) was performed as described previously.[5] All ACTs were evaluated by two observers. For each ACT, the Utrecht score was calculated: the Ki67 proliferation index + 4 if ≥ 33% of cells have clear/vacuolated cytoplasm, and + 3 if necrosis was present. The ACTs were classified according to their Utrecht score as low risk of recurrence tumors (LRTs; < 6 in Utrecht score) or moderate/high risk of recurrence tumors (MHRTs; ≥ 6 in Utrecht score).

Quantitative RT-PCR

Tissues were snap-frozen within 10 minutes after adrenalectomy (ACTs) or euthanasia (normal adrenals; NAs). RNA was isolated from tumor tissue or from the adrenal cortex of NAs with the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. The RNA concentrations were measured with Nanodrop (ND-1000; Isogen Life Sciences, Utrecht, The Netherlands) and cDNA was subsequently

Table 1 Primer pairs

Target gene		Primer sequence (5' > 3')	T^a (°C)
MC2R	For	TCA TGT GGT TTT GCC GGA AGA GAT	58.5
	Rev	AAT GGC CAG GCT GCA AAT GAA A	
INHA	For	AGG AGG ATG TCT CCC AGG C	67.0
	Rev	GTG TGG AAC CAC AGG TGG GC	
SF-1	For	AGG GCT GCA AGG GGT TTT TCA A	59.0
	Rev	CAT CCC CAC TGT CAG GCA CTT CT	
VAV2	For	CTG CTT ACT GGA GAT TCA GG	58.0
	Rev	GGG TCA TGT AGT TCT TCT CG	
PBX1	For	GCA TCA GTG CTA ATG GAG GT	60.3
	Rev	GCA GGT ATC AGA GTG AAC ACT G	
VNN1	For	AGT TGA AAC TGC TTC TAC C	61.6
	Rev	ACT TGA CAC CTG AAA TTC TC	
SOAT1	For	CAA CTA TCC TAG GAC TCC CAG	60.3
	Rev	CAT AGG ACC AGA ACG CGA	
PTTG1	For	GCC TCA GAT GAC ACC TAT CCA G	63.5
	Rev	AAG TTC CCT CTC CTC ATC AAG G	
RRM2	For	GAA GCT ACC TAT GGA GAA CGG	61.5
	Rev	GGT GTT TGA ACA TCA GGC AG	
TOP2A	For	CGG ACA CCT ACA TTG GCT	64.5
	Rev	GCA GCA TTG ACC AGA ATC TC	
MKI67	For	TCA GTT CCA GCA ATC CGA	61.5
	Rev	GCA GAG ATT CCT GTT TGC G	
CCND1	For	CCC GCA CGA TTT CAT TGA AC	61.0
	Rev	AGG GCG GAT TGG AAA TGA AC	
RAC1	For	TCC CTT ATC CTA TCC GCA AA	58.0
	Rev	ATG ATA GGG GTG TTG GGA CA	
BCL2	For	GGA TGA CTG AGT ACC TGA ACC	62.0
	Rev	CGT ACA GTT CCA CAA AGG C	

Primer pairs for RT-qPCR analysis. For, forward primer; Rev, reverse primer.

synthesized with the iScript cDNA Synthesis Kit (Bio-Rad, Veenendaal, The Netherlands) according to the manufacturer's instructions, and diluted to 1 ng/µl. Using SYBR-green Supermix (Bio-Rad) and a CFX384 Touch Real-Time PCR Detection System (Bio-Rad), the mRNA expression levels of 14 genes were analyzed with quantitative RT-PCR (RT-qPCR) analysis: melanocortin 2 receptor (*MC2R*), inhibin alpha subunit (*INHA*), *SF-1*, *VAV2*, pre-B-cell leukemia transcription factor 1 (*PBX1*), pantetheinase (*VNN1*), and sterol O-acyltransferase (*SOAT1*) (adrenal-associated genes); and *PTTG1*, ribonucleoside-diphosphate reductase subunit M2 (*RRM2*), *TOP2A, MKI67*, cyclin D1 (*CCND1*), Ras-related C3 botulinum toxin substrate 1 (*RAC1*), and *BCL2* (proliferation- or apoptosis-related genes). Primers (Table 1) were designed using Perl-primer software,[18] checked for secondary structure formation with the Mfold web server,[19] and ordered from Eurogentec (Maastricht, The Netherlands). Optimization and primer specificity confirmation were performed as described previously.[20]

For data normalization, the mRNA expression levels of 5 reference genes were analyzed: signal recognition particle receptor, succinate dehydrogenase complex subunit A, ribosomal protein S5, hypoxanthine-guanine phosphoribosyltransferase, and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta.[21–23]

Two technical replicates were analyzed for each sample. To exclude interference of genomic DNA, for each sample a control sample without reverse transcriptase was analyzed. GeNorm software[24] was used to analyze the reference genes' expression levels, which justified their use. The $2^{-\Delta\Delta Ct}$ method[25] was used to calculate the normalized relative expression of each target gene.

Statistical analyses

Because the data were not normally distributed, which was observed using the Shapiro-Wilk test, differences in mRNA expression levels between groups (NAs, LRTs, MHRTs) were analyzed with the Kruskall-Wallis test. Significant differences were analyzed post hoc with the Mann-Whitney U-test with Bonferroni correction.

For survival analyses, the ACT was considered to be the cause of death when the dog was euthanized due to recurrence of hypercortisolism, resulting from metastases or regrowth of the ACT. Survival times were recorded as the time between adrenalectomy and euthanasia due to recurrence (event occurred), or the time between adrenalectomy and the time of censoring (event did not occur). Dogs were censored when they died from an unrelated cause, were still alive at the end of the study, or were lost to follow-up. The Cox proportional hazards model was used for univariate survival analyses. To calculate optimal cut-off values, receiver operating characteristic (ROC) curves were used. To obtain clear results, only dogs with evidently good or bad prognoses were include for ROC curves: dogs that had recurrence and were euthanized within 30 months after adrenalectomy were included in the positive group (n = 9), and dogs that had no recorded recurrence and lived for at least 30 months after adrenalectomy were included in the negative group (n = 13). The cut-off value with the highest Youden index (sensitivity + specificity – 1) was selected as the optimal cut-off value. Survival times were subsequently calculated using the Kaplan-Meier product-limit method, and the log-rank test was used to determine whether a difference in survival times between groups was significant.

P-values < 0.05 were considered significant. All statistical analyses were performed with SPSS Statistics for Windows (Version 24.0, IBM Corp, Armonk, NY, USA).

Results

Cases

The ACTs of 40 dogs were included in the study. Dog breeds included Jack Russel Terrier (4), Labrador Retriever (4), Dachshund (3), Maltese (2), Schnauzer (2), White shepherd dog (2), mixed-breed (8), and 15 breeds that were represented once. Their median age at time of surgery was 10 years (range, 2 – 13 years) and their median body weight was 14 kg

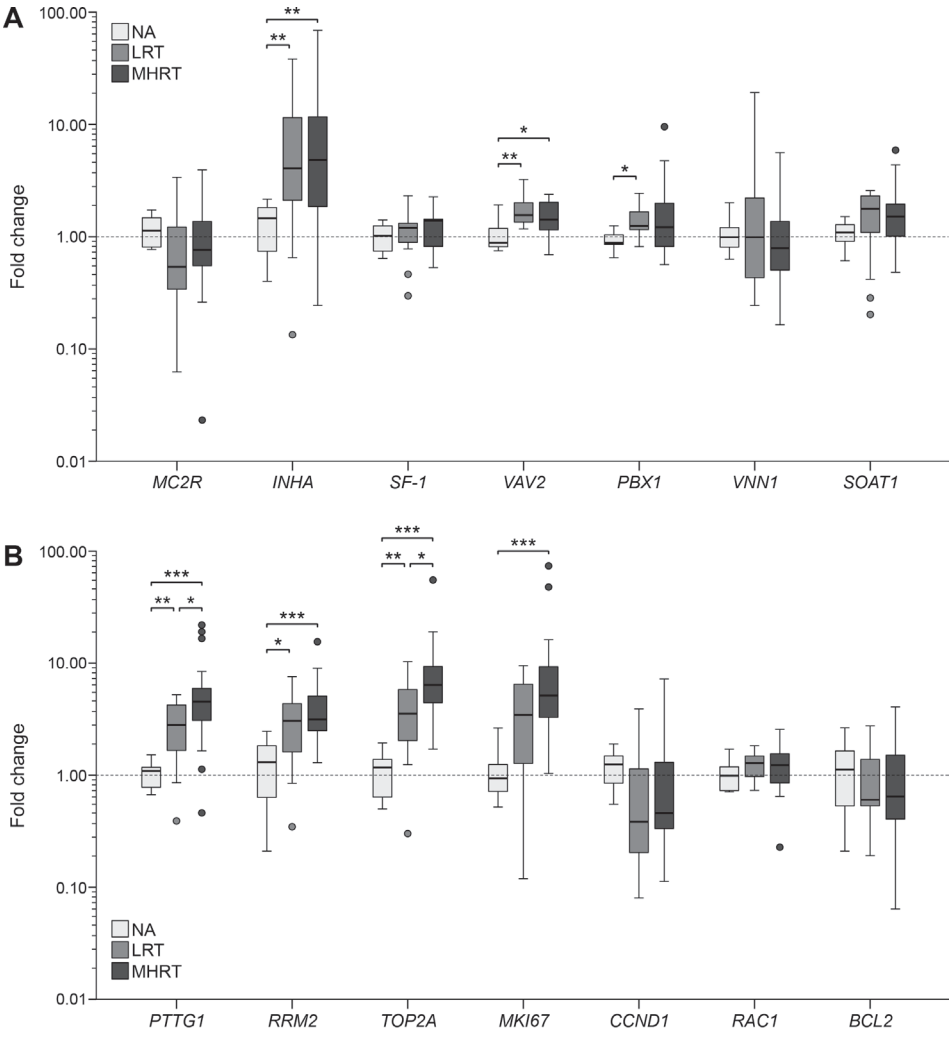

Figure 1 The mRNA expression levels in fold change (relative to the mean NA expression) of seven adrenal-associated genes (A) and seven proliferation- or apoptosis-associated genes (B). The dotted line represents the mean fold change of the NAs. The circles above and below the box plots indicate outliers. * P < .05, ** P < .01, *** P < .001. NA, normal adrenal (n = 11); LRT, low risk of recurrence tumor (n = 14); MHRT, moderate/high risk of recurrence tumor (n = 26).

(range, 4 – 45 kg). Nineteen dogs were female (11 spayed), and 21 were male (11 neutered). Based on histopathological analyses using the Utrecht score, 14 (35%) ACTs were classified as LRTs and 26 (65%) as MHRTs. The median survival time was not reached for the LRT group (recurrence observed in 1/14 dogs), and was 49.3 months (95% CI 12.9 – 85.6 months) for the MHRT group (recurrence observed in 14/26 dogs).

Of the 11 healthy dogs from which the adrenal glands were used as reference material, the dog breeds included Beagle (3) and mixed-breed (8). Their median age at time of euthanasia was 2 years (range, 1 – 5 years) and their median body weight was 23 kg (range, 10 – 26 kg). Eight dogs were female (none spayed) and 3 were male (none neutered).

Differential expression

In comparing the mRNA expression levels of the fourteen candidate genes between NAs, LRTs, and MHRTs, seven genes showed significant differences: *INHA, VAV2, PBX1, PTTG1, RRM2, TOP2A* and *MKI67*. Of these genes, the mRNA expression levels (Fig. 1) were significantly higher in LRTs compared to NAs for *PBX1*; in MHRTs compared to NAs for *MKI67*; in LRTs and in MHRTs compared to NAs for *INHA, VAV2*, and *RRM2*; and in LRTs and MHRTs compared to NAs but also in MHRTs compared to LRTs for *PTTG1* and *TOP2A*.

Survival analyses

In assessing the genes' association with survival times, the mRNA expression levels of three genes were significant: *SF-1, PTTG1,* and *TOP2A* (Table 2). When we classified the ACTs based on the genes' optimal cut-off values as calculated using ROC curves, the survival times were significantly different between the groups with high and low expression for all three genes (Fig. 2). For *SF-1*, the estimated median survival time of the group with low expression (fold change < 1.35; n = 23) was not reached, and of the group with high expression (≥ 1.35; n = 17) 16.9 months (95% CI, 1.5 – 32.2 months) (P < 0.001). For *PTTG1*, the estimated median survival time of the group with low expression (< 5.35; n = 30) was 56.6 months (95% CI, 52.1 – 61.1 months), and of the group with high ex-

Table 2 Survival analyses

Gene	Hazard ratio (95% CI)	P-value
MC2R	1.29 (0.77 – 2.16)	0.328
INHA	1.00 (0.96 – 1.03)	0.771
SF-1	8.23 (2.43 – 27.89)	*0.001**
VAV2	0.74 (0.27 – 2.08)	0.572
PBX1	1.14 (0.94 – 1.38)	0.187
VNN1	0.73 (0.44 – 1.23)	0.236
SOAT1	1.21 (0.85 – 1.72)	0.287
PTTG1	1.23 (1.11 – 1.37)	*<0.001**
RRM2	1.10 (0.92 – 1.32)	0.276
TOP2A	1.06 (1.02 – 1.11)	*0.005**
MKI67	1.02 (1.00 – 1.05)	0.080
CCND1	1.19 (0.82 – 1.72)	0.369
RAC1	2.65 (0.62 – 11.24)	0.188
BCL2	0.88 (0.47 – 1.65)	0.698

Univariate analyses performed with the Cox proportional hazards model. Significant P-values are indicated in italic font with an asterisk.

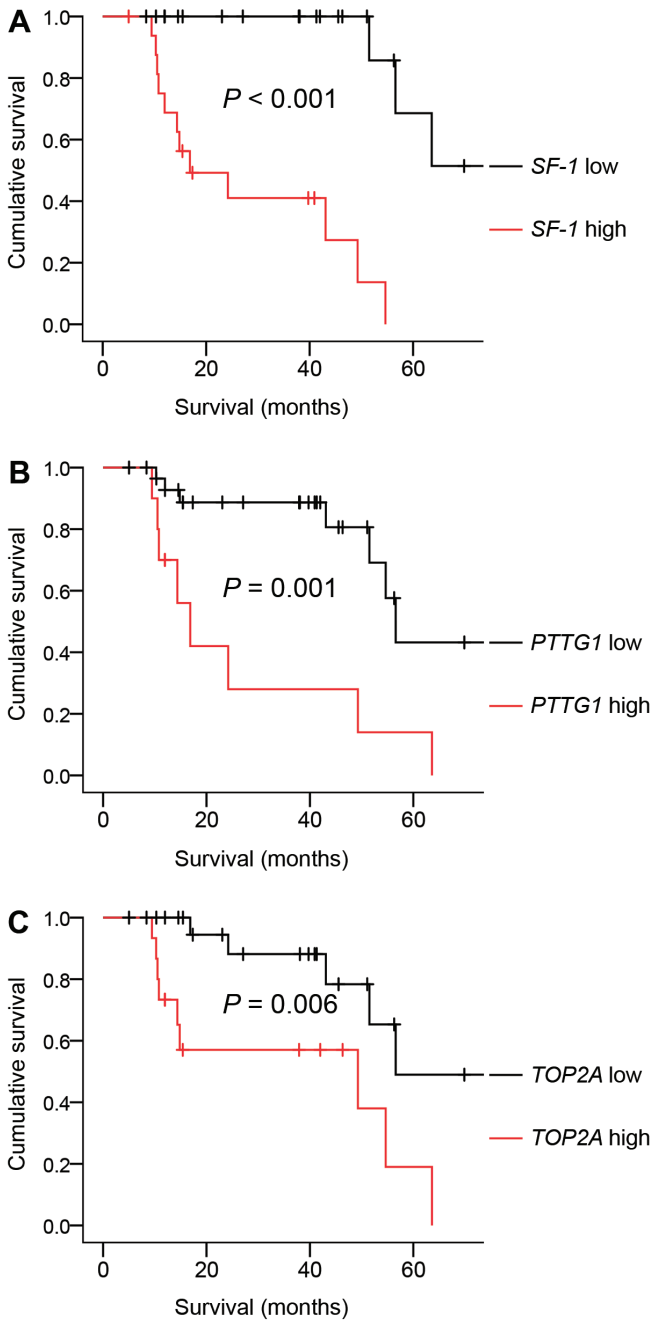

Figure 2 Survival stratified according to *SF-1* (A), *PTTG1* (B) and *TOP2A* (C) mRNA expression using Kaplan-Meier analyses. Dogs were classified as having (A) low (fold change < 1.35, n = 23) or high (≥ 1.35, n = 17) *SF-1* expression; (B) low (< 5.35, n = 30) or high (≥ 5.35, n = 10) *PTTG1* expression; and (C) low (< 6.31, n = 24) or high (≥ 6.31, n = 15) *TOP2A* expression. Tick marks indicate censored dogs. P-values indicate the significance of the difference between the respective groups as calculated with the log-rank test.

pression (≥ 5.35; n = 10) 16.9 months (10.7 – 23.0 months) (P = 0.001). For *TOP2A*, the estimated median survival time of the group with low expression (< 6.31; n = 24) was not reached, and of the group with high expression (≥ 6.31; n = 15) 49.3 months (95% CI, 47.6 – 61.7 months) (P = 0.006).

Discussion

In this study we identified seven genes that were differentially expressed between NAs and ACTs, of which *PTTG1* and *TOP2A* were also differentially expressed between LRTs and MHRTs. Moreover, we identified three genes of which high mRNA expression was significantly associated with poor survival: *SF-1, PTTG1*, and *TOP2A*.

SF-1 is an orphan nuclear receptor that is important in both adrenal development and steroidogenesis,[26] and the importance of *SF-1* gene dosage in adrenocortical tumorigenesis has been shown in multiple studies.[11,27,28] Interestingly, *SF-1* expression did not differ between LRTs and MHRT in the current study, nor between adrenocortical adenomas (ACAs) and adrenocortical carcinomas (ACCs) in a previous study,[10] which might appear to contradict its prognostic relevance. However, this is remarkably similar to the SF-1 expression pattern in human ACTs.[11] This apparent contradiction could be related to the difference in SF-1 function depending on the cellular context: in differentiated adrenocortical cells SF-1 mostly stimulates hormone production, whereas in fetal adrenal development SF-1 stimulates adrenal growth.[26,29] Possibly, ACA/LRT cells more closely resemble differentiated cells, and ACC/MHRT cells more closely resemble fetal cells, in which high SF-1 expression might provide a specific growth advantage.[11] Regardless of the mechanism, these results indicate that there might be room for improvement in the Utrecht score to refine prognostic classification.

The prognostic relevance of *SF-1* makes it an interesting therapeutic target. Compounds that can target SF-1 activity, called SF-1 inverse agonists, have been identified[30,31] and inhibited cell proliferation and steroid hormone production in vitro in human ACC cells.[32] We showed in a previous study that one SF-1 inverse agonist, compound #31, effectively inhibited cortisol production and SF-1 target gene expression in canine adrenocortical cells in vitro.[33] If SF-1 inverse agonists will be further developed for clinical use, this may have much potential to improve the prognosis of dogs with an ACC with high SF-1 expression.

VAV2 and *PBX1* were not associated with survival, but both were expressed significantly higher in LRTs compared to NAs, and *VAV2* also in MHRTs. In human ACCs, VAV2 is an important target of SF-1, and high VAV2 expression is a critical driver of cell invasion and strongly associated with poor prognosis.[15,34] PBX1 has been show to act synergistically with SF-1, and is also a target of SF-1.[35] The mRNA expression of both *VAV2* and *PBX1*

showed a weak but significant correlation with the *SF-1* mRNA expression (Spearman's rank correlation coefficient: VAV2 .341, P = .031; PBX1 .458, P = .003). The prognostic relevance of *VAV2* and *PBX1* could have been underestimated in the current study due to the relatively low number of cases, and it might be interesting to reanalyze the expression of these genes in a larger dataset.

PTTG1 is a securin that regulates sister chromatin separation during mitosis, and it plays a role in DNA repair, metabolism, senescence, apoptosis, and gene transcription.[36,37] PTTG1 is also a prognostic marker in human ACCs,[13,38] as well as in other tumor types in humans and dogs.[39–41] Several drugs have been shown to inhibit *PTTG1* expression, including BRAF, HDAC, Hsp90, and STAT3 inhibitors,[42–44] which could be interesting options to target PTTG1 in canine ACTs.

TOP2A is a nuclear enzyme that facilitates DNA unlinking, which is required for DNA replication and chromosome segragation.[45,46] TOP2A is predominantly associated with proliferating cells, which makes it an interesting therapeutic target in cancer. *TOP2A* is also a prognostic marker in human ACCs and other tumor types.[14,40] Several TOP2A inhibitors are therefore successfully used in the clinic as anticancer drugs.[45,46] The chemotherapy protocol that is most effective in human ACCs is the combination of etoposide, doxorubicin, and cisplatin with mitotane (EDP-M).[47] Of these, etoposide and doxorubicin are topoisomerase II inhibitors,[48] and a recent study showed that the level of *TOP2A* expression in human ACCs is predictive of the response to the EDP-M chemotherapy protocol.[14] In view of the prognostic relevance of *TOP2A* in canine ACTs, topoisomerase II inhibitors could be an interesting treatment to improve the prognosis of dogs with high intratumoral *TOP2A* expression.

Although *RRM2* was not significant in the survival analyses, its expression was significantly higher in ACTs compared to NAs. RRM2 is a rate-limiting enzyme that is essential for DNA synthesis and replication, and overexpression of *RRM2* is associated with poor prognosis in several cancer types.[49–53]

Interestingly, PTTG1, TOP2A, and RRM2 are all regulated by the transcription factor E2F1.[54–56] E2F1 is an activator within the mammalian E2F family that binds to promoters of genes that are important for S-phase progression in the cell cycle, which initiates DNA replication.[57] Recently, a peptide was identified that binds tightly to the E2F1 promoter consensus sequence. Coupled to penetratin, this peptide was cytotoxic to tumors that overexpress activating E2Fs.[58] Future developments in inhibitors of E2F1 activity could be interesting for canine MHRTs, since this approach might affect the expression levels of PTTG1, TOP2A, and RRM2.

INHA was included as a candidate gene because loss of *INHA* expression has been detected in a small subset of human ACCs, and could indicate a tumor suppressor role of INHA

in adrenocortical carcinogenesis.[59] *INHA* expression varied greatly within the ACTs, and although not significantly associated with survival, it was significantly higher in ACTs compared to NAs. In contrast, a recent study in human ACTs reported no significant differences in *INHA* expression between NAs and ACTs.[59] However, in this recent study the human ACTs were included irrespective of their hormonal production status, while in our study only cortisol-secreting ACTs were included. INHA is associated with cortisol and androgen production,[60,61] which might explain its relatively high expression levels in ACTs compared to NAs in the current study. Additionally, this difference in expression could be explained by the fact that in normal adrenal glands, INHA is not expressed in the zona glomerulosa but only in the zona fasciculata and zona reticularis.[61] We used the entire adrenal cortex of normal adrenal glands, including the zona glomerulosa, as reference material in our study, which could have contributed to the observed difference in expression. In line with our findings, dogs with ACTs have higher serum inhibin concentrations than healthy dogs or dogs with pheochromocytomas, which means that INHA can be used as diagnostic marker.[62]

In healthy adrenocortical cells, steroidogenesis is initiated by the binding of ACTH to the MC2R, which activates the cAMP – protein kinase A signaling pathway.[63] The MC2R can therefore be regarded as a marker for differentiated adrenocortical cells. *MC2R* expression varied within the ACTs but was not significantly different between NAs and ACTs nor was it associated with survival. In a previous study by our group, the *MC2R* mRNA expression was significantly lower in ACCs compared to NAs.[20] In our current study, the ACT classification is based on the recently developed histopathological Utrecht score, which is different from the previous study and might explain the different result found in this study.

SOAT1 expression was not significantly different in ACTs compared to NAs. Nevertheless, the *SOAT1* mRNA expression pattern is important information, since SOAT1 was recently reported to be a target of the drug mitotane (o,p'-DDD).[64] The expression of SOAT1 could therefore be indicative of the expected efficacy of mitotane, as it is in humans,[64] although more studies are required to determine the minimal SOAT1 expression required for efficacy of mitotane. ATR-101, also a SOAT1 inhibitor, is currently being studied as a potential treatment for humans with an ACC.[65,66] It has recently also been administered to dogs with hypercortisolism, the majority having pituitary-dependent hypercortisolism, in which it significantly decreased ACTH-stimulated cortisol concentrations.[67] ATR-101 could be an interesting treatment for dogs with an ACT, and further studies are warranted to determine whether there is a minimal degree of SOAT1 expression required for its effect.

In conclusion, in this study we have identified several genes that are part of the molecular signature of malignancy in canine ACTs. Most apparent molecular markers of malignancy were *SF-1, PTTG1* and *TOP2A*. These findings can be used to refine prognostic prediction, but also offer substrate for future studies, where important prognostic markers could be targeted for new treatment options. If in the future drugs for multiple targets are available, treatment of dogs with high risk of recurrence could be based on their ACT's molecular malignancy profile, thereby moving towards personalized treatment.

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Chapter

9

Summarizing Discussion and Key Points

Hypercortisolism, often referred to as Cushing's syndrome, is one of the most common endocrine disorders in dogs. It is caused by an ACTH-secreting pituitary tumor (pituitary-dependent hypercortisolism; PDH) in ~80-85% of cases, and by a cortisol-secreting adrenocortical tumor (ACT) in ~15-20% of cases.[1]

For the treatment of canine hypercortisolism, pharmacotherapy is often used. The use of trilostane, a steroidogenesis inhibitor, is advised in dogs with PDH, and the use of either trilostane or mitotane, an adrenocorticolytic drug, is advised in dogs with a cortisol-secreting ACT. The advantage of trilostane is that it is effective and usually well tolerated, whereas its main disadvantage is that it does not selectively inhibit cortisol production.[2,3] The main adverse effect that can occur is transient hypocortisolism (shortage of glucocorticoids), possibly combined with or followed by complete hypoadrenocorticism (shortage of both glucocorticoids and mineralocorticoids).[4] Moreover, trilostane only controls the clinical signs of hypercortisolism and does not inhibit growth of the pituitary tumor or the ACT. The advantage of mitotane is that it can destroy ACT cells, whereas its main disadvantage is that it can cause serious side-effects, and due to its cytotoxicity it should not be used when there are young children or pregnant women in the household.[1] Both medical treatment options therefore have disadvantages.

To expand the currently available range of treatment options, new medical treatment options with high selectivity and tolerability are desired. However, to search for new treatment targets, more knowledge is required on normal canine adrenal physiology and on prognostic markers of canine ACTs. Therefore, the aims of this thesis were:

1. To determine which enzymes are involved in canine steroidogenesis, as to discover which enzymes can be used as targets for selective cortisol inhibition (**Chapter 3**)
2. To test the efficacy of potential future treatment options *in vitro* in a canine primary adrenocortical cell culture model (**Chapters 4, 5** and **6**)
3. To identify prognostic indicators in canine cortisol-secreting ACTs, which can help to select dogs with high risk of recurrence after adrenalectomy that could benefit from adjuvant therapy, and which could help to identify potential future treatment targets (**Chapters 7** and **8**)

The results, implications, limitations, and future perspectives of the studies included in this thesis are discussed in this chapter.

(Inhibiting) Steroidogenesis

Because the end-products of the adrenocortical mineralocorticoid (aldosterone) and glucocorticoid (cortisol) pathways are the same in dogs as in humans, it was assumed that the steroidogenic pathway would also be identical. However, it had never been studied whether this was really the case. **In Chapter 3** we therefore investigated the zonal expression of steroidogenic enzymes in the canine adrenal cortex.

In humans the last step in aldosterone synthesis is catalyzed by aldosterone synthase (CYP11B2) in the zona glomerulosa (zG), whereas the last step in cortisol synthesis is catalyzed by 11β-hydroxylase (CYP11B1) in the zona fasciculata (zF).[5,6] In contrast, cows, pigs, sheep, birds and amphibians have only CYP11B1 to catalyze the last steps in both aldosterone and cortisol synthesis.[7–11] When we looked for the canine variants of these genes in the NCBI database, we found the sequence of a single canine *CYP11B* gene which was called *CYP11B2*, and that a large sequence gap was present near this gene. Based on these findings we wondered: do dogs have both *CYP11B1* and *CYP11B2*, or has the only canine *CYP11B* gene mistakenly been identified as *CYP11B2*? **Chapter 3** provides four clues that help us to answer this question.

(1) In zG and zF regions that were specifically isolated using Laser Microdissection, we analyzed the mRNA expression of the so-called *CYP11B2* in normal canine adrenals. We found that there was no difference in the so-called *CYP11B2* gene expression between the zG and zF, which was our first clue.

(2) Because the CYP11B gene duplicates in humans and mice lie very close to each other,[6] we hypothesized that if another canine *CYP11B* gene copy is present, its sequence could be hidden in the sequence gap near the so-called *CYP11B2* gene. We ordered Bacterial Artificial Chromosomes (BAC) clones that contained either only the so-called *CYP11B2*, or both the so-called *CYP11B2* and the sequence gap. Using Southern blot combined with a *CYP11B* probe from exons 3 to 4, we concluded that no additional *CYP11B* gene copy was present in the sequence gap, which was our second clue.

(3) Of the BAC containing the sequence gap, a draft sequence of unordered contigs can be found in the NCBI database. On these contigs, exon 1, half of exon 2, and exon 9 could be identified, in which exon 2 was quickly followed by exon 9. Exons 3-8 were not found on any of the contigs belonging to the sequence gap, which was our third clue.

(4) The amino acid sequence of a CYP11B protein can predict what the functional activity of that enzyme might be.[12] Analysis of the known canine CYP11B amino acid sequence suggested that it has aldosterone synthase activity, but also a higher 11β-hydroxylase activity than the human CYP11B2. This implies that the known canine CYP11B has aldosterone synthase activity but is also efficient in cortisol production, which was our fourth clue.

None of these clues provide definitive proof, and arguments can be devised to dispute each clue individually. To provide definitive proof, future studies could include *in vitro* cell culture experiments with knock-out or knock-in of the canine so-called *CYP11B2* gene, and determine its functional activity in terms of aldosterone and cortisol production. Moreover, improvements in sequencing and annotation of the complete canine genome could provide the definitive answer in the future. However, all the clues in **Chapter 3** combined strongly suggest that dogs have only one functional *CYP11B* gene (Fig. 1), which has mistakenly been identified as *CYP11B2* in the NCBI database.

Because CYP11B enzyme expression does not appear to explain zone-dependent steroidogenesis in dogs, this must depend on a different zone-specific enzyme. In humans, 17α-hydroxylase/17,20-lyase (CYP17A1) is known to be absent in the zG because it is not required for aldosterone production, whereas it is present in the zF and zona reticularis (zR) because it is required for cortisol and androgen production.[13] In contrast, in the adrenal cortex of adult rats, mice, and rabbits, CYP17A1 is not expressed, which is why corticosterone and not cortisol is the end-product of their glucocorticoid pathway.[14,15] As shown in **Chapter 3** we found that in the canine adrenal cortex, *CYP17A1* mRNA is expressed significantly higher in the zF than in the zG. Immunohistochemistry gave similar results, since it showed that CYP17A1 protein expression was limited to the zF and zR, and was not present in the zG. The zone-specific CYP17A1 expression is therefore an important factor in canine steroidogenesis, because it is absent in the zG in which aldosterone is produced, but present in the zF and zR in which cortisol and androgens, respectively, are produced.

The zone-specific expression of CYP17A1 makes it an interesting target for selective inhibition of cortisol production, without concurrent inhibition of aldosterone production. Consequently, we went in search of CYP17A1 inhibitors. The candidate that we found was abiraterone acetate (AA). AA is a potent CYP17A1 inhibitor that has been approved in 2011 in Europe and the United States to inhibit androgen production in patients with metastatic castration-resistant prostate cancer (mCRPC).[16,17] Androgens stimulate prostate tumor growth in patients with mCRPC, which is why CYP17A1 inhibition is an important treatment strategy in these patients.[18] AA is generally well tolerated in these patients, but understandably also induces hypocortisolism, which necessitates coadministration of glucocorticoids to reduce the associated side effects.[19] Although the induction of hypocortisolism is unintentional in patients with mCRPC, this offers interesting opportunities for

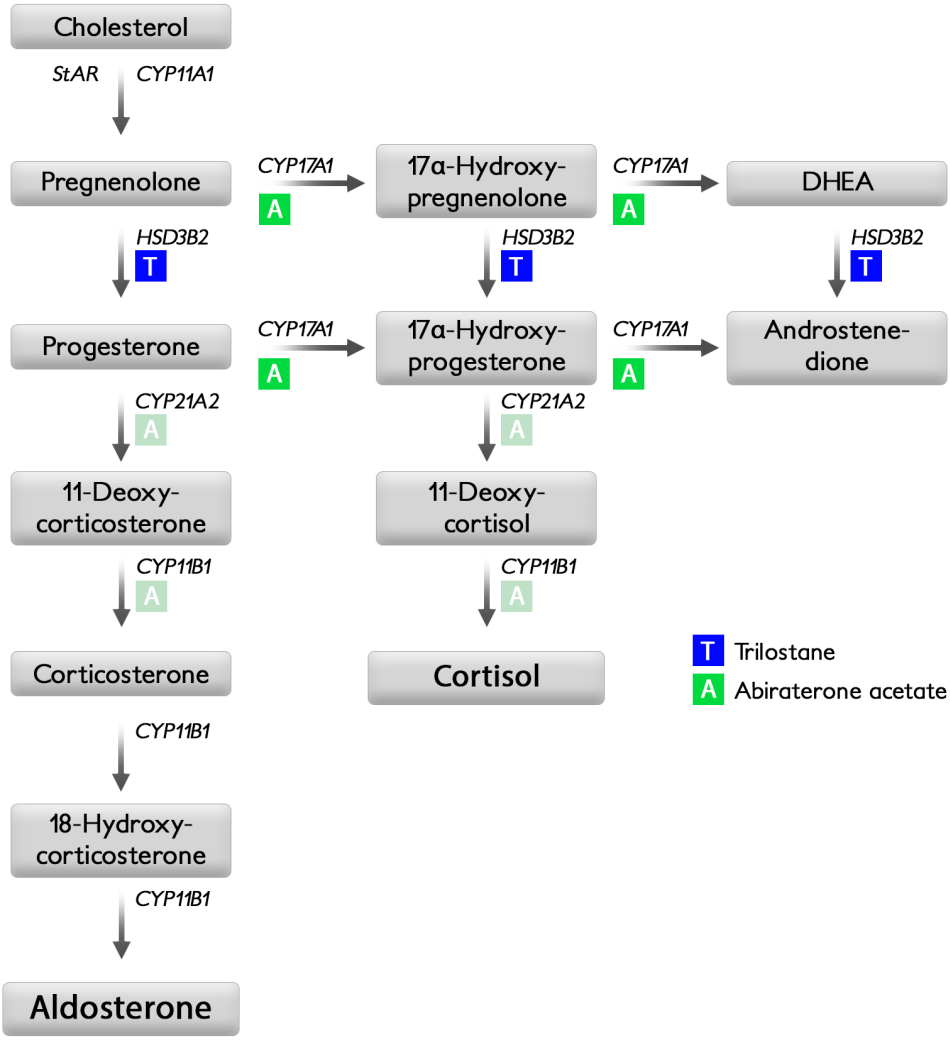

Figure 1 Canine adrenocortical steroidogenesis as proposed in **Chapter 3**. Main site of action of trilostane, the most commonly used medical treatment for canine hypercortisolism, indicated in blue. Main site of action of abiraterone acetate indicated in green, expected additional sites of action based on the results in **Chapter 4** indicated in light green.

the treatment of hypercortisolism. Therefore, in **Chapter 4** we evaluated the potential of AA as a future treatment option for canine hypercortisolism.

In line with the principles of the 3Rs (Replacement, Reduction and Refinement of laboratory animals), we decided to test AA's functionality on cultured canine primary adrenocortical cells. In **Chapter 4** we assessed how AA affects the hormone production, mRNA expression of steroidogenic enzymes, and cell viability in canine primary adrenocortical cell cultures (n = 9) under basal and ACTH-stimulated conditions. By measuring the con-

centration of 13 steroid hormones with liquid chromatography-tandem mass spectrometry (LC-MS/MS), we were able to determine specifically which steroid hormones in the adrenocortical pathway were affected by AA. The results of this study showed that AA potently inhibited glucocorticoid production, including cortisol, but did not affect aldosterone production. Moreover, as expected AA substantially inhibited the 17α-hydroxylase reaction catalyzed by CYP17A1, but to a lesser extent also the 21-hydroxlyase reaction catalyzed by CYP21A2 and the 11β-hydroxylase reaction catalyzed by CYP11B1 (Fig. 1). The inhibition of CYP21A2 by AA has been reported previously,[20,21] and might be partly or completely caused by the active metabolite Δ^4-abiraterone. This metabolite is known to be a CYP21A2 inhibitor,[22] and was abundantly present in the cell culture medium as indicated with LC-MS/MS. AA inhibited neither mRNA expression of steroidogenic enzymes nor cell viability.

Overall the results of **Chapter 4** seem very promising for AA as a future treatment option for canine hypercortisolism. However, an important limitation of this cell culture system is that we cannot mimic the feedback system of the hypothalamic-pituitary-adrenal (HPA) axis. *In vivo*, the decreased glucocorticoid production could result in increased plasma ACTH concentrations, as also occured in human patients with mCRPC that were treated with AA.[23] In these patients this in turn markedly increased steroids upstream of CYP17A1 that can exert mineralocorticoid activity (e.g., 11-deoxycorticosterone), which can lead to the mineralocorticoid excess syndrome. Because of subsequent suppression of the renin-angiotensin-aldosterone system, aldosterone itself did not increase. For technical reasons we measured 11-deoxycorticosterone only in four cell cultures in our study, but despite the moderate inhibition of AA on CYP21A2, AA increased the mean 11-deoxycorticosterone concentration under ACTH-stimulated conditions. This effect might be greater *in vivo* when ACTH secretion increases due to a low cortisol concentration. However, whereas the goal of treatment in patients with mCRPC is to completely block androgen production, the goal of treatment in dogs with hypercortisolism would be to achieve normocortisolism. Nevertheless, the potential increase in ACTH secretion *in vivo* warrants caution. While these mineralocorticoid excess effects could be countered by using other drugs such as aldosterone receptor antagonists (e.g., spironolactone, eplerenone) or epithelial sodium channel inhibitors (e.g., amiloride),[24,25] this is not convenient and would not be practical for long-term use. *In vivo* experiments are therefore warranted to analyze the effects of AA in the presence of a functional HPA axis in dogs, with a particular focus on how AA affects plasma 11-deoxycorticosterone concentrations.

These *in vivo* experiments will also elucidate whether AA is superior to trilostane. Even if AA is not superior but nonetheless efficacious, it would be beneficial to have more medical treatment options, so that a good alternative is present for dogs that do not respond well to trilostane. Moreover, in the treatment of humans with Cushing's syndrome the use of combination therapy is on the rise, in which the use of two or even more drugs could increase the efficacy while limiting the toxicity of the individual drugs.[26]

One of the disadvantages of trilostane is that it can induce necrosis in the adrenal cortex.[27,28] This necrosis is presumably not caused by trilostane directly, but by the previously mentioned increased plasma ACTH concentrations resulting from loss of negative feedback.[2,29] If this is indeed the case, necrosis could also be induced when using AA. If this effect of ACTH could be blocked, this would have many advantages to include in combination therapy, since this would counter many of the disadvantages of trilostane and of the potential risks of AA. More importantly, blocking the effect of ACTH would be an interesting monotherapy for dogs with PDH. The receptor for ACTH in the adrenal cortex is the $G_{s\alpha}$-protein coupled melanocortin 2 receptor (MC2R). The MC2R is one of five melanocortin receptors, MC1R-MC5R, which are all activated by melanocortin peptides derived from the precursor pro-opiomelanocortin.[30] Although multiple melanocortin peptides can bind to the other melanocortin receptors, unique for the MC2R is that it binds only ACTH.[31,32] A selective and potent MC2R antagonist would therefore be an exquisite treatment option to selectively block ACTH-induced detrimental effects, either as combination therapy or as monotherapy for dogs with PDH. In **Chapter 5** we had the opportunity to test two peptide compounds, BIM-22776 (#776) and BIM-22A299 (#299), to determine whether they were potential MC2R antagonists *in vitro*.

We analyzed whether the peptide compounds could block the ACTH-induced effects on cortisol production and on mRNA expression of *MC2R* itself, the melanocortin 2 receptor accessory protein (*MRAP*), and steroidogenic enzymes, of canine primary adrenocortical cells. The results presented in Chapter 5 show that both #776 and #299 are effective MC2R antagonists, but that #299 is most potent. Compound #299 significantly and substantially inhibited ACTH-stimulated cortisol production and mRNA expression of *MC2R, MRAP* and steroidogenic enzymes, and therefore seems a promising future treatment option for canine PDH. However, three important caveats should be kept in mind.

Firstly, the selectivity of the compound for the MC2R relative to the other melanocortin receptors is of paramount importance. Since the other melanocortin receptors have a variety of functions in other tissue types, inadvertently antagonizing or agonizing these receptors could result in many unwanted side-effects.[33] In previous attempts for the creation or isolation of MC2R antagonists either no data for MC2R selectivity was reported or the peptides used also had some agonist and/or antagonistic effects on other melanocortin receptors.[34–37] In the study described in **Chapter 5** we have only analyzed the compounds' potency and not their selectivity. Future studies to determine their selectivity could include cell lines transfected with the canine *MC2R* and *MRAP* and the other melanocortin receptors, stimulated with ACTH and with α-MSH, and analysis of the compounds' effect on all melanocortin receptors with radioligand binding assays and cAMP-assays.

Secondly, other MC2R antagonists that were previously shown to be effective *in vitro*[37] showed disappointing results in rats *in vivo* in a subsequent study,[38] which could also be the case with compounds #776 and #299.

Thirdly, peptides are known to have low bioavailability, a short half-life and rapid plasma clearance *in vivo*. However, a great deal of attention is being paid to solving these problems to improve the applicability of proteins and peptides as medical treatment options,[39] which could also be beneficial for the future of peptide MC2R antagonists.

Although the theory of MC2R antagonists and the potential of #299 are hopeful, more *in vitro* and *in vivo* studies are required before this approach can be used in clinical practice. Interestingly, a new compound has recently been identified that was reported to be specific to the murine MC2R, and studies are currently underway to study its effectiveness *in vivo*.[40] It would be interesting to determine this compound's effect on the canine melanocortin receptors.

For the previously described potential future treatment options, a clear disadvantage is that although they might effectively inhibit cortisol production, they are not expected to inhibit the growth of either the pituitary tumor or the ACT. For cases with PDH, the development of for example dopamine/somatostatin chimeras could be an interesting future treatment approach to target the pituitary tumor itself.[41,42] For cases with an ACT, factors that provide an adrenal-specific growth advantage would be interesting treatment targets, as well as factors that are associated with a poor prognosis. When an ACT has been surgically removed, prognostic factors could also help to select dogs with high risk of recurrence that could benefit from adjuvant therapy after surgery. **Chapters 7** and **8** are therefore focused on identifying prognostic factors in canine ACTs.

(Targeting) Prognostic Factors

Assessing the risk of recurrence after adrenalectomy is usually based on histopathology. However, the histopathological parameters that are mostly used in the assessment of canine ACTs have not been linked to survival times,[43] and some studies did not observe a significant difference in survival times of dogs after adrenalectomy based on their histopathological diagnosis.[44,45] Moreover, some of these parameters are known to have high interobserver variability in the assessment of human ACTs.[46,47] We therefore wanted to assess whether we could identify histopathological parameters that have low intra- and interobserver variability, and are associated with poor survival after adrenalectomy. With these parameters we then aimed to establish a scoring system. The results in **Chapter 7** show that of all histopathological parameters scored, including the Ki67 proliferation index, less than 25% had moderately good or better agreement scores. This indicates that the majority of the parameters previously used are indeed difficult to score. In the hope of improving this, we analyzed the prognostic relevance only of parameters that had sufficient intra- and interobserver agreement scores. We weighted the significant variables based on their rounded hazard ratios in multivariate analyses, after which we ended up with the following score (Fig. 2): the Ki67 proliferation index, + 4 if ≥ 33% of tumor cells

have clear/vacuolated cytoplasm, + 3 if necrosis is present. Using cut-off values of 6 and 11, we could distinguish three groups that had significantly shorter survival times with increasing Utrecht scores. We propose to classify ACTs with a score of < 6 as having low risk of recurrence, with a score of ≥ 6 to < 11 as having moderate risk of recurrence, and with a score of ≥ 11 as having high risk of recurrence.

Because increased cell proliferation and tumor hypoxia are important hallmarks of cancer,[48] it is not surprising that a high Ki67 proliferation index and the presence of necrosis mark malignancy, as they also do in human ACTs.[46,49] Why the high percentage of clear/vacuolated cytoplasm marks malignancy is less clear, especially since the opposite is true in human ACTs according to the commonly used histopathological Weiss score, in which the presence of clear cytoplasm in less than 25% of the tumor is a negative prognostic factor.[50] A possible explanation is that these vacuoles indicate the presence of lipid droplets in which much cholesterol is stored, which functions as substrate for the production of steroid hormones, and therefore indicates higher steroid hormone production. In human ACTs, cortisol production is known to be a negative prognostic indicator,[51,52] which would be in line with this hypothesis. We were unable to test this hypothesis in **Chapter 7** because the diagnosis of hypercortisolism of the included dogs was made with different methods and hormone assays, but this would be interesting to determine in future studies. Moreover, it could be worthwhile to stain the tumors with a lipid staining, e.g., the Oil red O stain, which would help to visualize the lipid droplets. Such a staining could also be analyzed using digital analysis, with ImageJ software to calculate lipid intensity scores as described previously.[53] Whether such lipid intensity scores also have prognostic value would have to be determined, but if this is the case then this could improve the repeatability and reliability of the scores even further.

Irrespective of the mechanism behind the included parameters, the Utrecht score appears to be a useful tool to assess the prognosis of dogs with cortisol-secreting ACTs. However, the method used in **Chapter 7** has based its calculations on this specific subset of dogs and pathologists. The score will therefore have to be validated in a different population

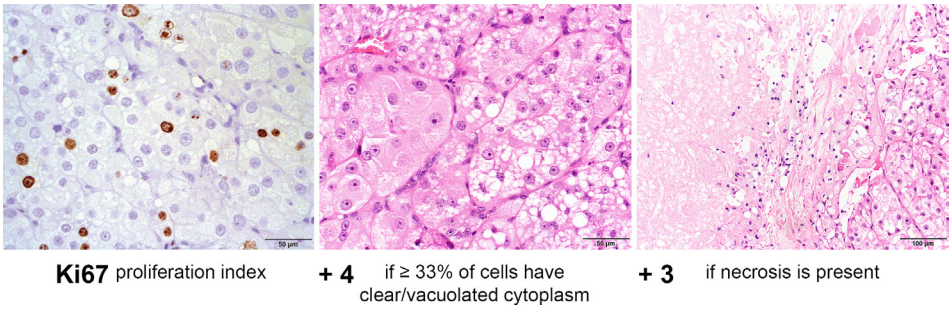

Figure 2 The Utrecht score in canine adrenocortical tumors as presented in **Chapter 7**: Ki67 proliferation index, + 4 if ≥ 33% of tumor cells have clear/vacuolated cytoplasm, + 3 if necrosis is present.

and with the involvement of multiple (veterinary) pathologists. Moreover, it would be interesting to determine whether the score is also useful for the assessment of non-secreting ACTs, i.e., incidentalomas. In addition to evaluating its prognostic relevance in these tumors, this could also help us to determine whether the importance of clear/vacuolated cytoplasm is indeed related to increased hormone production. Consequently, if that is the case, then incidentalomas on average would have lower Utrecht scores and are expected to be less malignant overall, as is the case in humans.[51,52]

In humans, in addition to the classical form of ACTs, other histopathological subtypes have also been identified, including adrenocortical oncocytic and myxoid tumors. To our knowledge, these subtypes have not been described in dogs. In humans, adrenocortical oncocytic neoplasms (ACONs) account for approximately 10% of all adrenocortical tumors. Although the majority of ACONs are benign, certain attributes that are intrinsic to these tumor types are assessed as malignant parameters in the Weiss score, e.g., eosinophilic character, elevated Fuhrman grade, and usually diffuse architectural structure.[54] The Weiss score is therefore not suitable for diagnostic and prognostic assessments of ACONs, and an adjusted Lin-Weiss-Bisceglia score has been developed for this tumor type.[55] The recently developed Helsinki score has also been tested on ACONs, and turns out to be applicable,[49] although ACONs with high Helsinki scores possibly remain less malignant than conventional ACCs with high Helsinki scores.[54] For the myxoid subtype the Weiss score is also unsuitable, but in contract to ACONs, the malignancy of myxoid ACTs often gets underestimated. Myxoid ACCs are comparable to or even more malignant than conventional ACCs, and the presence of a myxoid ACT is in itself highly suspicious for the presence of malignancy.[56] Interestingly, a recent case report describes an aldosterone-producing adrenocortical carcinoma (ACC) with myxoid differentiation in a cat.[57] If these subtypes also exist in canine ACTs, it would be interesting to determine whether the Utrecht score is also useful in these cases.

Of the 19 dogs with recurrence described in **Chapter 7**, the median survival time was 16.9 months (95% CI, 10.8 – 49.3 months). If these dogs would have been classified as having a high risk of recurrence, they could have received adjuvant treatment post-operatively which might have improved their survival times. Moreover, if molecular markers that are associated with malignancy and thus a high risk of recurrence could be identified, this could give more insight into which molecular pathways are useful to target for future treatment options. In **Chapter 8** we therefore evaluated the mRNA expression of 14 candidate genes and assessed whether they were associated with the histopathological Utrecht score and/or with the dogs' survival times.

We identified seven genes that were differentially expressed between normal adrenals (NAs) and ACTs, of which pituitary tumor-transforming gene-1 (*PTTG1*) and topoisomerase II alpha (*TOP2A*) were also differentially expressed between low risk of recurrence tumors (LRTs) and moderate/high risk of recurrence tumors (MHRTs). In survival analyses, the mRNA expression of three genes was significantly associated with poor

survival: *PTTG1, TOP2A*, and Steroidogenic factor-1 (*SF-1*). All three have also been reported to have prognostic value in human ACTs.[58–60]

Fitting to its name, *PTTG1* has also been reported to be a prognostic markers in pituitary tumors of dogs with PDH.[61] This is especially interesting since it would mean that if compounds could be identified that efficiently target PTTG1 in dogs, this could be useful for all dogs with spontaneous Cushing's syndrome. Several drugs have been shown to inhibit *PTTG1* expression, including inhibitors of BRAF, HDAC, Hsp90, and STAT3,[62–64] which would be interesting treatment options to test in future studies.

Although PTTG1 as a target might seem like a long way off, TOP2A as a target could be feasible in a nearer future. The most efficacious chemotherapy protocol for human ACCs already includes two topoisomerase II inhibitors: etoposide and doxorubicin.[60,65] In case of doxorubicin, this is already being used to improve the survival times of dogs with lymphoma and other cancer types.[66–68] In case of etoposide, intravenous etoposide administration in dogs induced acute hypersensitivity reactions, probably associated with the vehicle used for the parental formulation (polysorbate-80),[69] whereas oral administration had low bioavailability.[70] A recent study focused on intravenous etoposide phosphate administration in dogs with multicentric lymphoma, in which no hypersensitivity reactions were reported.[71] Etoposide phosphate is a water-soluble pro-drug of etoposide that does not require polysorbate-80 as a vehicle, and gets rapidly and extensively converted to etoposide by phosphatases in serum and tissues.[72] A phase II study in dogs is currently ongoing for the use of etoposide phosphate in dogs with lymphoma,[71] which might also prove to be useful for dogs with an ACT. In view of the prognostic relevance of *TOP2A* in canine ACTs, topoisomerase II inhibitors could be an interesting treatment to improve the survival of dogs with high intratumoral *TOP2A* expression after adrenalectomy.

SF-1, also known as adrenal 4-binding protein (Ad4BP) and encoded by the *NR5A1* gene, is an orphan nuclear receptor that plays an essential role in the development and function of steroidogenic tissues.[73] The results in **Chapter 8** show that although *SF-1* mRNA expression was not significantly different in LRTs compared to MHRTs, or in ACTs compared to NAs, its expression was significantly associated with survival times, both on a continuous scale and when groups were classified as having high or low expression. These results might appear to contradict each other, but are remarkably similar to the SF-1 expression pattern in human ACTs.[58] This apparent contradiction could be related to the difference in SF-1 function depending on the cellular context: in differentiated adrenocortical cells SF-1 mostly stimulates hormone production, whereas in fetal adrenal development SF-1 stimulates adrenal growth.[74,75]

How SF-1 function and activity is regulated is complex and not yet completely understood, but includes phospholipids binding inside its hydrophobic pocket, a large array of transcription factors and coregulators, and posttranscriptional modifications such

as phosphorylation and sumoylation.[73,76] The difference in SF-1 function depending on the cellular context could be related to GATA-4 and/or GATA-6, both members of the GATA family of transcription factors that regulate gene expression, development, and proliferation in a variety of tissues. In the human embryonic adrenal cortex, *GATA-4* and *GATA-6* are both present, but postnatally *GATA-4* gets downregulated while *GATA-6* remains expressed.[77] Multiple studies have shown that the expression of *GATA-4* is higher in ACCs than in adrenocortical adenomas,[78–80] with one exception reporting the opposite.[81] Although one study reports that *GATA-6* expression is not affected by tumorigenesis,[80] other studies report that *GATA-6* on average is downregulated in ACCs, but remains high in hormone-producing ACCs.[79,82] Both GATA-4 and GATA-6 are functionally linked to SF-1,[82,83] so hypothetically, SF-1 in the presence of GATA-6 could increase hormone production, while SF-1 in the presence of GATA-4 could increase cell proliferation. For future studies it would be interesting to determine the expression of GATA-4 and GATA-6 in the canine ACTs samples.

The prognostic relevance of *SF-1* and its importance in adrenocortical proliferation implies that inhibition of SF-1 activity would be an interesting treatment approach for dogs with ACTs. Moreover, SF-1 is also an important factor for dogs with PDH, since ACTH increases cortisol secretion, at least partly, by increasing SF-1 activity.[84] Inhibition of SF-1 activity would therefore be an interesting treatment strategy for dogs with PDH and for dogs with an ACT. Luckily, inhibitors of SF-1 activity have been identified. Therefore, in **Chapter 6** we investigated the efficacy of three SF-1 inhibitors, SID7969543 (IsoQ A) and its two analogs #31 and #32, on cultured canine primary adrenocortical cells. Based on their assumed mode of action, these SF-1 inhibitors were referred to as SF-1 inverse agonists.[85]

We employed canine primary adrenocortical cells of NAs, under basal and ACTH-stimulated conditions, and of three ACTs. The results showed that of these three compounds, #31 most effectively inhibited cortisol production and SF-1 target gene expression in canine primary adrenocortical cells of NAs, under both basal and ACTH-stimulated conditions. In the ACTs, #31 resulted in different degrees of cortisol inhibition. We hypothesized that this might be related to the ACTs' *SF-1* mRNA expression levels. Although the three cultures were too few to statistically test this hypothesis, the ACT with the greatest *SF-1* expression showed most cortisol inhibition by #31 *in vitro*, whereas the ACT with lowest *SF-1* mRNA expression showed least cortisol inhibition. This suggests that the efficacy of SF-1 inverse agonists is SF-1 dosage dependent, as also reported in a different study.[85]

In ACTs, especially those with relatively high *SF-1* expression, an additional advantage of SF-1 inverse agonists would be to also inhibit cell proliferation. We therefore also assessed whether the compounds inhibited cell viability of normal adrenocortical and ACT cells, which was not the case. However, the proliferation rate of the cells used in this culture system is low, meaning that three days of incubation is probably not enough to see a sig-

nificant effect on inhibition of cell proliferation when this is assessed using a cell viability assay. Additional experiments to analyze their effect on proliferation in vitro could include longer incubation times, or the use of a different assay, e.g., an EdU proliferation assay. For the latter, because we cannot exclude the presence of co-culture of the adrenocortical cells with other cell types such as fibroblasts, this would require the addition of an adrenocortical marker to ensure that we're only analyzing the proliferation rate of the adrenocortical cells. To increase proliferation rates, the addition of adrenal-specific growth factors such as N-terminal pro-opiomelanocortin[86] could be useful, although this might increase proliferation independent of SF-1. Moreover, a larger ACT sample size would be required, since the effect of SF-1 inverse agonists on proliferation could be different in ACTs than in NAs, especially considering its dual function on steroidogenesis and proliferation. In a previous study, all three compounds significantly inhibited the *SF-1* overexpression-induced cell proliferation in a human ACC cell line.[85] Considering that #31 significantly inhibited cortisol production and SF-1 target gene expression as described in **Chapter 6**, it seems likely that it could also inhibit proliferation in ACTs with high *SF-1* expression. Based on the differences in potency between the different compounds tested, #31 is an interesting candidate for further development into a drug that can be used *in vivo*. This could be useful to inhibit cortisol production in dogs with PDH and dogs with a cortisol-secreting ACT, but could potentially also inhibit tumor growth in dogs with an ACT.

Science's next top model*

Primary adrenocortical cell culture

In **Chapters 4, 5**, and **6** we have used canine primary adrenocortical cell culture to test the efficacy of different compounds. This is a novel model that, to our knowledge with only one exception,[87] has not been described previously. Such a cell culture system has both advantages and disadvantages.

The main advantage of using a cell culture system is that the potential of future treatment options can be evaluated preclinically as a screening method, which reduces the number of laboratory animals required. The results of **Chapters 4, 5**, and **6** show that the adrenocortical cells in culture produce hormones and express mRNA of steroidogenic enzymes and steroidogenic factors, which shows that they remain differentiated and functional adrenocortical cells *in vitro*. Moreover, they respond substantially to ACTH stimulation, as apparent by increased cortisol secretion, but also 17-hydroxyprogesterone, 11-deoxycortisol, corticosterone, and androstenedione secretion as shown in **Chapter 4**, as well as increased mRNA expression of steroidogenic enzymes, *MC2R, MRAP,* and *SF-1* as shown in **Chapters 5** and **6**, and a decrease in *DAX1* expression as shown in **Chapter 6**. This provides useful tools to analyze how new compounds affect hormone production and gene

* Term used by courtesy of Honors Program Veterinary Medicine students 2017-2018

expression of adrenocortical cells. Additionally, this culture system allows us to examine the effects of new compounds on a cellular level without interference of the *in vivo* HPA feedback system, which can provide different mechanistic insights. An advantage of primary cell culture compared to an immortal cell line is that it is more heterogeneous which is more representative of the adrenal cortex *in vivo*, and could therefore be biologically more relevant.

While the absence of the HPA feedback system can be an advantage, it can also be considered as a disadvantage, since this complicates the prediction of the effect and possible complications of a compound *in vivo*. A culture system also does not provide any information on pharmacokinetics and pharmacodynamics, which will still have to be evaluated *in vivo*. Although we expect that the more heterogeneous nature of primary cell culture in vitro will be a better predictor of *in vivo* functionality, we do not yet know whether this is really the case. Disadvantages of primary cell culture compared to an immortal cell line include limited gene transfection efficiencies[88] and slow proliferation rates.

Implicit in its name, a model is merely an approximation of and not identical to the system it represents. Nonetheless, canine primary adrenocortical cell culture is a valuable technique and a useful tool for a first screening, and therefore a good starting point to test a potential future treatment option.

Dogs as animal models for Cushing's syndrome in humans

The chapters in this thesis are mostly focused on improving the treatment and prognostication of Cushing's syndrome in dogs. However, as already mentioned in **Chapter 4**, canine Cushing's syndrome shares many similarities with its human counterpart. These similarities can be observed in at least four areas: the etiology, since in both species the most common causes are PDH or an ACT, with also a similar distribution; the clinical presentation, including clinical signs such as abdominal obesity, weight gain, fatigue and muscle atrophy; treatment options, which in both species include surgery, steroidogenesis inhibitors and mitotane; and similar adrenocortical zonation patterns.[1,26,89,90] In addition, the incidence is approximately a thousand times higher in dogs than in humans,[91,92] which can generate substantial amounts of tumor tissue for *in vitro* research. Other advantages of the dog as an animal model include a shorter overall lifespan and more rapid disease progression compared to humans, which is convenient for *in vivo* studies.[93] Compared to, e.g., *in vivo* mouse models, spontaneous canine models have the advantages of an intact immune system, shared environmental risk factors, inter-individual and intratumoral heterogeneity, and the possibility for development of recurrent or resistant disease.[94]

The studies described in **Chapters 3, 7** and **8** of this thesis have contributed to our understanding of the canine normal adrenal cortex and of ACTs, providing more insight on the suitability of the dog as an animal model for Cushing's syndrome. The results of **Chapter 3** showed that steroidogenesis of the dog is not identical to that of humans, meaning that

if we want to evaluate the effect of a specific CYP11B1 or CYP11B2 inhibitor, the dog is not a useful model. However, to evaluate the effect of a CYP17A1 inhibitor, as we did *in vitro* in **Chapter 4**, the dog would be an excellent model and superior to, e.g., adult rats or mice, who do not express CYP17A1 in their adrenal cortex.[15,95] **Chapter 7** showed that not all prognostically relevant histopathological parameters in dogs are the same in humans, but there are also important similarities, i.e., necrosis and the Ki67 proliferation index are both very important in the assessment of both canine and human ACTs.[46] The importance of *SF-1, PTTG1* and *TOP2A* as prognostic markers became clear in **Chapter 8**. Interestingly, all three are also important prognosticators in human ACTs,[58–60] meaning that for research on inhibiting these markers or their associated pathways in ACTs, dogs would be a useful model.

Just as with an *in vitro* model, an *in vivo* animal model is not identical to the system it represents. Of paramount importance when choosing the optimal animal model is knowing what you want to target, and whether that target is present and known to have the same importance in that animal model. For the compounds tested in this thesis, i.e., AA, MC2R antagonists, and SF-1 inverse agonists, the compounds' targets are present and seem to be as relevant in dogs as in humans. Dogs would therefore be useful animal models to further test these compounds in *in vivo* studies, with the important additional advantage that these results can be used to improve treatment of both human and canine Cushing's syndrome.

General conclusions

The studies included in this thesis have contributed to prognostication of canine ACTs, the identification of potential treatment targets, and knowledge on the efficacy and mechanism of action of potential new treatment options. The findings presented in this thesis can be used for future studies, with the ultimate goal to increase the range of medical treatment options that are available for both canine and human Cushing's syndrome. These future treatment options could prove to be superior to commonly used drugs, or provide an alternative for humans or dogs that do not respond well to currently available treatment options. Moreover, multiple drugs could be employed as combination therapy, in which the use of two or even more drugs could increase the efficacy while limiting the toxicity of the individual drugs. In cases with an ACT, if in the future drugs for prognostically relevant targets are available, treatment of dogs with high risk of recurrence could be based on their ACT's molecular malignancy profile, thereby moving towards personalized treatment.

Key Points

(Inhibiting) Steroidogenesis

- Dogs seem to have only one functional CYP11B enzyme, hereafter referred to as CYP11B1, that catalyzes the last steps in both cortisol and aldosterone production.
- The canine adrenal cortex expresses CYP17A1 in the zF and zR, which is likely to be the only enzyme that is required for cortisol but not aldosterone production.
- The CYP17A1 inhibitor AA effectively reduces glucocorticoid secretion of canine primary adrenocortical cells, but did not affect aldosterone production. AA did not only inhibit CYP17A1, but to a lesser extent also CYP21A2 and CYP11B1. *In vivo* studies are warranted to assess the potential of AA as a future treatment option for canine Cushing's syndrome.
- Two peptide compounds, #776 and #299, were effective antagonists of the MC2R in vitro in canine primary adrenocortical cells, of which #299 was the most potent. More studies are required on their selectivity for the MC2R compared to other melanocortin receptors.

(Targeting) Prognostic Factors

- Less than 25% of all histopathological parameters that we assessed in canine ACTs had moderately good or better agreement scores. Of these, the Ki67 proliferation index, the percentage of clear/vacuolated cytoplasm, and the presence of necrosis were associated with poor survival of dogs with a cortisol-secreting ACT after adrenalectomy.
- Using the histopathological Utrecht score with cut-off values of 6 and 11, dogs could be divided in three groups, which had significantly shorter survival times with increasing Utrecht scores.
- Of fourteen target genes, the mRNA expression levels of three genes were significantly associated with survival: *SF-1, PTTG1*, and *TOP2A*. Based on these findings, it could be useful to treat dogs with high intratumoral *TOP2A* expression with topoisomerase inhibitors such as doxorubicin and etoposide to improve their survival times after adrenalectomy. Inhibition of SF-1 activity would be an interesting treatment approach to inhibit both hormone production and adrenocortical cell proliferation in dogs with Cushing's syndrome.
- Of three SF-1 inverse agonists tested in canine primary adrenocortical cells, #31 most potently inhibited cortisol production and SF-1 target gene expression. In ACT cells, #31 resulted in different degrees of cortisol inhibition, which could be related to their *SF-1* mRNA expression levels. #31 is an interesting candidate for further development into a drug that can be used *in vivo*, which could be useful to inhibit cortisol production in all dogs with Cushing's syndrome, and to potentially also inhibit tumor growth in dogs with an ACT that has high *SF-1* expression.

Science's next top model

- Cultured canine primary adrenocortical cells produce hormones, express mRNA of steroidogenic enzymes and steroidogenic factors, and respond to ACTH stimulation. Canine primary adrenocortical cell culture is therefore a valuable model to evaluate the efficacy and mechanism of action of new compounds as a first screening, so that *in vivo* testing is reserved only for compounds with high potential.
- Because the adrenal cortex expresses CYP17A1 in both dogs and humans, because the MC2R is essential in both canine and human PDH, and because *SF-1, PTTG1*, and *TOP2A* have prognostic relevance in both canine and human ACTs, dogs with spontaneous Cushing's syndrome are useful animal models to evaluate the potential of inhibitors of any of these factors.

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Chapter

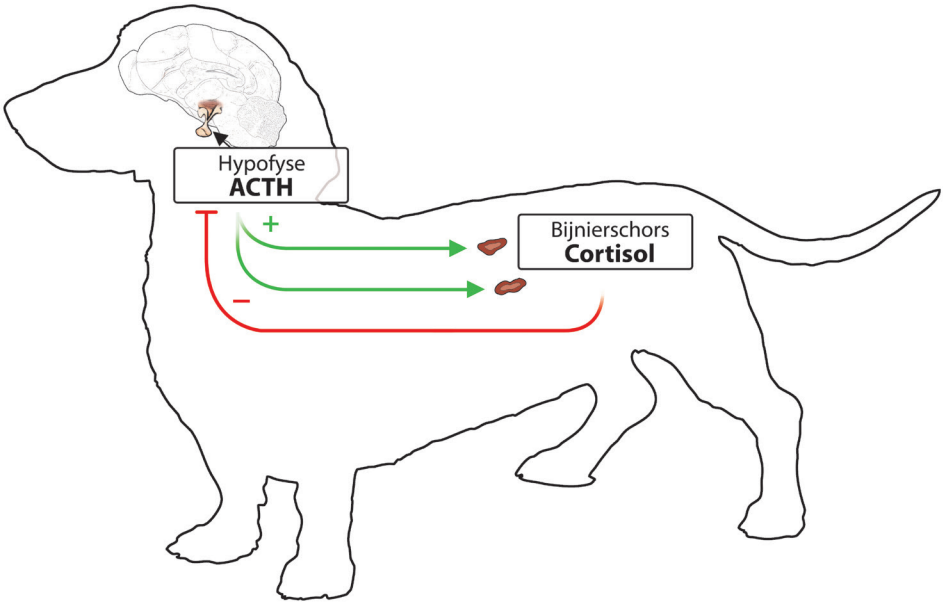
10

Samenvatting en Conclusies

De bijnieren zijn twee gepaarde organen die in de buurt van de nieren liggen en verschillende hormonen produceren. Een bijnier bestaat uit twee delen: het bijniermerg, dat onder andere adrenaline produceert, en de bijnierschors, die onder andere cortisol produceert. Cortisol is een hormoon dat essentieel is voor het lichaam om met stress om te kunnen gaan, het zorgt bijvoorbeeld voor een hogere suikerspiegel in je bloed zodat je tijdelijk beter kan presteren. Normaliter is de productie en afgifte van cortisol goed gereguleerd (Fig. 1): een stressfactor zorgt er voor dat de hypofyse adrenocorticotroop hormoon (ACTH) afgeeft aan het bloed, ACTH geeft een signaal aan de bijnierschors dat de cellen cortisol moeten produceren en afgeven, en cortisol geeft vervolgens weer een signaal terug aan de hypofyse dat er genoeg cortisol aanwezig is, en er minder ACTH aangemaakt hoeft te worden. Cortisol remt dus zijn eigen productie, dit noemen we ook wel een negatieve feedbackloop. In sommige gevallen is de cortisol afgifte echter niet meer goed gereguleerd, en wordt er chronisch teveel cortisol aangemaakt. Deze aandoening wordt hypercortisolisme genoemd, ook bekend als het syndroom van Cushing, en kan voorkomen bij zowel mensen als honden.

Het syndroom van Cushing is bij honden een van de meest voorkomende afwijkingen aan het hormoonstelsel. In ongeveer 80 tot 85% van de gevallen wordt dit veroorzaakt door een tumor in de hypofyse die chronisch teveel ACTH produceert, en dus constant signalen aan de bijnierschors afgeeft dat er cortisol moet worden geproduceerd. Dit noemen we hypofyse-afhankelijk hypercortisolisme (HAH). In ongeveer 15 tot 20% van de gevallen wordt het syndroom van Cushing veroorzaakt door een cortisol-producerende bijnierschorstumor (BST). In dit geval blijft de bijnierschors cortisol produceren zonder dat het daarvoor een signaal van ACTH nodig heeft.

Voor de behandeling van het syndroom van Cushing bij honden kan de hypofyse- of bijnierschorstumor chirurgisch worden verwijderd. Deze ingreep is echter niet zonder risico's, niet in iedere kliniek mogelijk en niet geschikt voor iedere patiënt. Daarom wordt vaak medicatie gebruikt om de cortisol productie te remmen. In het geval van HAH wordt aangeraden om trilostane te gebruiken, een middel dat een bepaalde stap in de productie van cortisol remt. In het geval van een cortisol-producerende BST kan naast trilostane ook mitotane worden gebruikt, een middel dat bijnierschorscellen afbreekt. Beide middelen hebben echter ook nadelen. Trilostane remt namelijk niet alleen de productie van cortisol



Figuur 1 Versimpelde weergave van de regulatie van cortisol afgifte: door een stressfactor geeft de hypofyse adrenocorticotroop hormoon (ACTH) af aan het bloed, dit geeft een signaal aan de bijnierschors dat de cellen cortisol moeten produceren, en cortisol remt vervolgens zijn eigen productie door de afgifte van ACTH door de hypofyse te remmen.

maar ook van andere hormonen die in de bijnierschors worden gemaakt, en mitotane is een vrij toxisch middel dat veel bijwerkingen kan geven. De huidige behandelingsmogelijkheden voor het syndroom van Cushing zijn samengevat in **Hoofdstuk 2**.

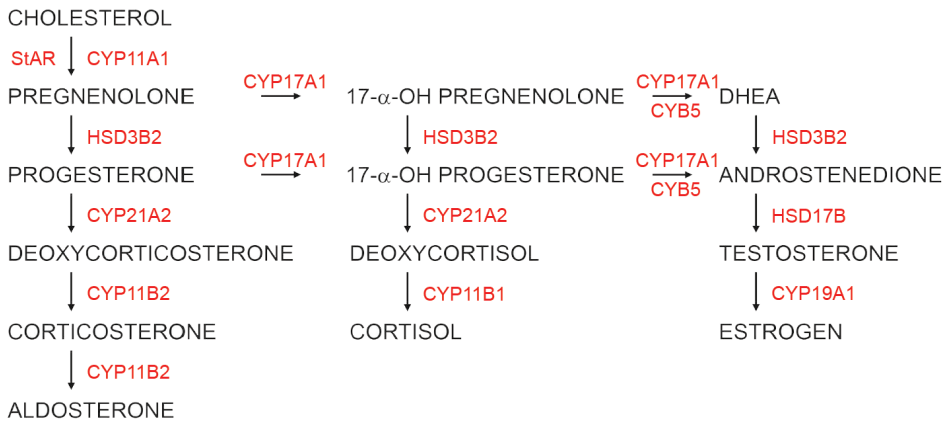
Om het aantal medicamenteuze opties te vergroten is het gewenst om over nieuwe middelen te beschikken met hoge selectiviteit en weinig bijwerkingen. Om te weten welke factoren we hiervoor moeten aangrijpen, is het nuttig om meer te weten over de stappen die nodig zijn voor de productie van cortisol. In het geval van een BST is het daarnaast nuttig om te weten welke factoren in een BST zorgen voor een slechte prognose na een operatie. De drie hoofddoelen van dit proefschrift waren daarom:

1. Vaststellen welke enzymen er nodig zijn voor de productie van cortisol en de andere bijnierschorshormonen, zodat we weten welk enzym we zouden kunnen remmen om selectief cortisol te remmen (**Hoofdstuk 3**)
2. Het testen van potentieel nieuwe behandelingsmogelijkheden in gekweekte bijnierschorscellen van honden (**Hoofdstukken 4, 5** en **6**)
3. Identificeren van prognostische factoren bij honden met een cortisol-producerende BST, zodat we honden kunnen selecteren die een hoog risico hebben op het terugkeren van de tumor na operatie en daarom mogelijk baat hebben bij nabehandeling, en omdat het remmen van specifieke prognostische factoren zou kunnen bijdragen aan het verlengen van de overlevingsduur (**Hoofdstukken 7** en **8**)

Hormoonproductie (remmen)

De bijnierschors bestaat uit drie zones: de buitenste zona glomerulosa (zG), de middelste zona fasciculata (zF) en de binnenste zona reticularis (zR). In iedere zone worden er andere hormonen geproduceerd: in de zG wordt aldosteron geproduceerd, een hormoon dat belangrijk is voor het zoutgehalte in het bloed en daarmee de bloeddruk; in de zF wordt het stresshormoon cortisol geproduceerd; en in de zR worden voorgangers van geslachtshormonen geproduceerd. Het gezamenlijke startpunt voor ieder hormoon is cholesterol. Met behulp van verschillende enzymen, de steroïdogenese enzymen, wordt cholesterol in meerdere tussenstappen omgezet in de verschillende eindproducten.

Als we de productie van cortisol ten opzichte van aldosteron in mensen nader bekijken, zien we in Figuur 2 dat het enzym 17α-hydroxylase/17,20-lyase (CYP17A1) nodig is voor de productie van cortisol maar niet voor aldosteron. Daarnaast worden de laatste stappen in aldosteron productie gekatalyseerd door het enzym aldosteron synthase (CYP11B2), terwijl de laatste stap in cortisol productie wordt gekatalyseerd door 11β-hydroxylase (CYP11B1). Omdat de eindproducten van deze paden in honden hetzelfde zijn als in mensen, werd gedacht dat ook dezelfde enzymen hiervoor nodig zouden zijn. Het was echter nooit onderzocht of dit echt het geval was. In sommige dieren, bijvoorbeeld in koeien, schapen en varkens, is er slechts één CYP11B enzym dat de laatste stappen in zowel aldosteron als cortisol productie katalyseert, dit enzym wordt dan CYP11B1 genoemd. De doelstelling van **Hoofdstuk 3** was om de expressie van steroïdogenese enzymen in de verschillende zones van de bijnierschors te onderzoeken, en te bepalen of honden één of twee CYP11B enzymen hebben.



Figuur 2 Schema van hormoonproductie in mensen. Hormonen zijn in zwart aangegeven en de enzymen die de verschillende reacties katalyseren in rood.

Als eerste zochten we in de NCBI database de genen voor *CYP11B1* en *CYP11B2* in honden op. Gek genoeg konden we wel een gen vinden dat *CYP11B2* genoemd werd, maar geen gen dat *CYP11B1* genoemd werd. Naast het zogenoemde *CYP11B2* gen was er een groot gat in de DNA sequentie zichtbaar, wat wil zeggen dat de genetische code in dat gebied niet goed is afgelezen en geïdentificeerd. Omdat de verschillende *CYP11B* genen in mensen en muizen dicht bij elkaar liggen, leek het ons waarschijnlijk dat als de hond een tweede *CYP11B* gen heeft, dat het in dit sequentie gat zou zitten. Vervolgens hebben we twee stukjes DNA besteld: één waar alleen het zogenoemde *CYP11B2* gen op zat, en één waar zowel het *CYP11B2* gen als het sequentie gat in zat. We ontworpen een probe voor een *CYP11B* gen, oftewel een stukje DNA wat aan een *CYP11B* gen zou moeten binden. Met de Southern blot techniek hebben we gekeken of deze probe alleen aan het zogenoemde *CYP11B2* gen zou binden of ook aan een gen in het sequentie gat, waarna we konden concluderen dat er geen extra *CYP11B* gen aanwezig was in het sequentie gat.

Ook hebben we met een Laser Microdissectie microscoop specifiek heel kleine stukjes gesneden uit de zG en zF. Hiermee konden we specifiek RNA isoleren uit deze zones, en met behulp van primers die specifiek het zogenoemde *CYP11B2* gen herkennen en vermenigvuldigen, konden we bekijken of dit gen verschillend tot uiting komt in de zG ten opzichte van de zF. Dit was echter niet het geval, terwijl als het CYP11B2 enzym alleen voor aldosteron productie gebruikt zou worden, dan zouden we verwachten dat het enkel in de zG tot uiting komt. Hoewel het geen definitief bewijs is, konden we door deze bevindingen, in combinatie met een aantal andere aanwijzingen, concluderen dat de hond slechts één *CYP11B* gen heeft, wat ten onrechte *CYP11B2* genoemd wordt in de NCBI database.

Aangezien er dus geen verschil lijkt te zijn in de types CYP11B enzymen die belangrijk zijn voor de laatste stappen in aldosteron en cortisol productie bij honden, moet er wel een ander enzym zijn dat zone-specifiek tot expressie komt. Een logische kandidaat hiervoor is het enzym CYP17A1, wat bij mensen wel nodig is voor cortisol maar niet voor aldosteron productie. In **Hoofdstuk 3** bleek dat CYP17A1 inderdaad bij honden selectief tot expressie komt in de zF en zR, maar niet in de zG. CYP17A1 lijkt dus het enige enzym te zijn dat belangrijk is voor de productie van cortisol maar niet van aldosteron. Dit is belangrijke informatie, omdat we nu weten dat als we selectief de cortisol productie willen remmen zonder de aldosteron productie te remmen, dat CYP17A1 hier een goed doelwit voor is. Daarom gingen we op zoek naar remmers van CYP17A1, waarbij we uitkwamen op abirateron acetaat (AA), een middel dat bij mensen gebruikt wordt om de productie van geslachtshormonen te remmen. In deze patiënten remt AA echter niet alleen productie van geslachtshormonen, maar ook van cortisol. Hoewel de remming van cortisol productie bij deze patiënten ongewenst is, biedt het juist mogelijkheden voor het gebruik van AA voor de behandeling van het syndroom van Cushing. In **Hoofdstuk 4** hebben we daarom geëvalueerd of AA potentie heeft als behandelingsoptie voor het syndroom van Cushing.

Om te zien wat het effect van AA op de bijnierschors van honden is, zonder daar veel proefdieren voor nodig te hebben, hebben we de werking van AA getest op gekweekte bijnierschorscellen van honden. Met een techniek genaamd vloeistofchromatografie – tandem massaspectrometrie konden we de concentratie van veel verschillende bijnierschorshormonen meten, waardoor we specifiek konden zien welke hormonen reageerden op AA en hoe. We zagen hierbij dat AA inderdaad cortisol productie aanzienlijk remt, maar geen effect had op de aldosteron productie. Daarnaast zagen we dat AA zoals we hadden verwacht het CYP17A1 enzym remt, maar in mindere mate ook het 21-hydroxylase (CYP21A2) en CYP11B1 enzym. De resultaten in **Hoofdstuk 4** zijn over het algemeen veelbelovend, maar het nadeel aan een kweeksysteem is dat we de feedbackloop zoals weergegeven in Figuur 1 niet kunnen nabootsen. *In vivo*, dus in het lichaam, zal een verlaagde cortisol productie zorgen voor een verhoogde ACTH productie die de bijnierschors aanzet om meer hormonen te maken. De productie van cortisol wordt geblokkeerd door de sterke remming op CYP17A1, maar ondanks de lichte remming op CYP21A2 is het goed mogelijk dat er meer 11-deoxycorticosteron aangemaakt zal worden, een hormoon met een zelfde soort werking als aldosteron, met de mogelijke nadelen (zoals een verhoogde bloeddruk) die daar bij horen. Of dit inderdaad zo gebeurt en in welke mate zal moeten blijken in toekomstige *in vivo* studies.

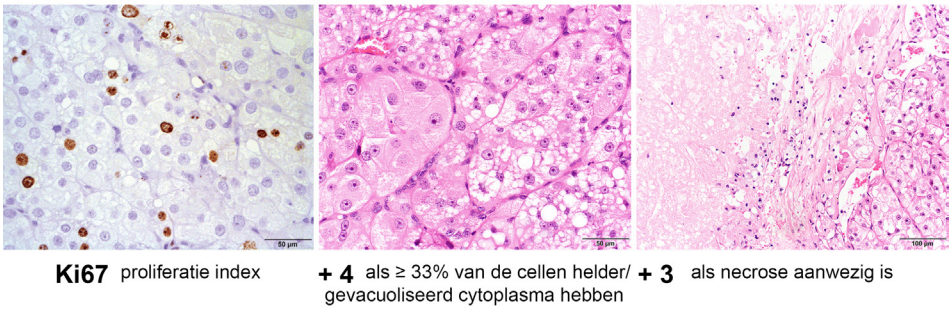
Zoals hierboven genoemd zou de verhoogde ACTH productie problemen kunnen geven bij toediening van AA. Dit is ook een van de problemen bij het veel gebruikte middel trilostane. Als dit effect van ACTH zou kunnen worden geblokkeerd zou dit zeer nuttig zijn om toe te voegen als combinatie therapie. Belangrijker nog, omdat de overmatige ACTH productie de oorzaak van de cortisol overmaat is in honden met HAH, zou het blokkeren van dit effect een zeer interessante monotherapie zijn voor honden met HAH. Het binden van ACTH aan zijn receptor in de bijnierschors, de melanocortin 2 receptor (MC2R), zet de cascade in gang die ervoor zorgt dat cortisol wordt geproduceerd. Daarom hebben we in **Hoofdstuk 5** de effectiviteit van twee antagonisten (stoffen die de receptor blokkeren) van de MC2R getest: BIM-22776 (#776) en BIM-22A299 (#299). Ook hiervoor hebben we gekweekte bijnierschorscellen van honden gebruikt. We zagen dat beide stoffen zorgden voor een significante remming van ACTH-gestimuleerde cortisol productie en genexpressie van steroïdogenese enzymen, en #299 was daarbij het meest potent. De MC2R is niet de enige melanocortin receptor in het lichaam, er zijn namelijk nog vier anderen (MC1R en MC3R – MC5R) die verschillende functies in het lichaam hebben. We willen daarom niet dat de MC2R antagonisten ook een effect hebben op die andere receptoren, maar dat ze enkel op de MC2R gaan zitten. In toekomstige studies is het dus belangrijk om de selectiviteit van de stoffen te bepalen. Hoewel meer ontwikkelingen en studies nodig zijn, lijkt het gebruik van MC2R antagonisten een zeer interessante toekomstige therapie.

Een nadeel van de hierboven genoemde medicamenteuze opties, is dat hoewel ze effectief kunnen zijn in het remmen van de cortisol productie, we niet verwachten dat ze ook de groei van een hypofyse tumor of BST zullen remmen. In het geval van een BST zou het in-

teressant zijn om doelwitten aan te pakken die specifiek in de bijnier zorgen voor een groei voordeel, en/of doelwitten die geassocieerd zijn met een slechte prognose na operatie. Factoren die de prognose van de hond na operatie kunnen voorspellen kunnen daarnaast ook helpen om honden te selecteren die een hoog risico hebben op het terugkeren van de tumor, en die mogelijk baat hebben bij aanvullende therapie. **Hoofdstukken 7** en **8** zijn daarom gericht op het identificeren van prognostische factoren in BST's.

Prognostische factoren (aangrijpen)

Normaal gesproken wordt het inschatten van de kans op recidief, dus het terugkeren van de tumor, gebaseerd op histopathologie. De parameters die hierbij vaak door de patholoog worden bekeken hebben echter een hoge intra- en inter-waarnemer variabiliteit in het geval van humane BST's en zijn dus moeilijk te beoordelen. Daarnaast was er in sommige studies geen significant verschil in de overleving van de honden na operatie gebaseerd op de histopathologische diagnose. Wij wilden daarom evalueren of er parameters zijn die relatief makkelijk te beoordelen zijn en ook echt geassocieerd zijn met de overleving na operatie. Een veterinaire patholoog en een veterinaire patholoog in opleiding hebben bij tumoren van 50 honden 21 histopathologische parameters beoordeeld op twee verschillende tijdstippen. In **Hoofdstuk 7** kwam naar voren dat minder dan 25% van deze parameters voldoende makkelijk te beoordelen waren. Van deze parameters waren drie parameters significant geassocieerd met overleving na de operatie, en uiteindelijk kwamen we daarmee uit op het volgende scoresysteem, wat we de Utrecht score noemden: de Ki67 proliferatie index, + 4 punten als minstens 33% van de cellen helder/gevacuoliseerd cytoplasma (blaasjes in de cel) hebben, + 3 punten als er necrose aanwezig is (Fig. 3). Met afkapwaardes van 6 en 11 konden we met dit score systeem de honden indelen in drie groepen, waarbij de groepen met hogere Utrecht scores significant kortere overlevingstijden hadden dan de groepen met lagere scores. Om de Utrecht score te valideren zouden we in de toekomst moeten testen of dit in een andere groep honden ook werkt waarbij meerdere (veterinaire) pathologen de tumoren beoordelen. Ook zou het interessant zijn om te kijken of de Utrecht score ook werkt bij BST's die geen hormonen produceren.



Figuur 3 De Utrecht score in cortisol-producerende bijnierschorstumoren van honden zoals berekend in **Hoofdstuk 7**

Van de honden in **Hoofdstuk 7** die recidief hebben gekregen, was de mediane overlevingstijd na operatie ongeveer 17 maanden. Als direct na de operatie het hoge risico op recidief al was herkend, hadden aanvullende therapie mogelijk de overlevingstijd van deze honden kunnen verlengen. Als we daarnaast ook nog factoren kunnen identificeren die geassocieerd zijn met een hoog risico op recidief en die we met medicijnen zouden kunnen remmen, dan zouden we een gerichtere therapie toe kunnen passen. In **Hoofdstuk 8** hebben we daarom de genexpressie van 14 kandidaat genen geëvalueerd, en gekeken of ze geassocieerd waren met de histopathologische Utrecht score en/of met de overlevingstijden van de honden.

We hebben in **Hoofdstuk 8** zeven genen geïdentificeerd die hoger tot uiting kwamen in BST's ten opzichte van normale bijnieren. Daarnaast hebben we drie genen geïdentificeerd die significant geassocieerd waren met een slechtere overleving: Steroidogenic factor-1 (*SF-1*), pituitary tumor-transforming gene-1 (*PTTG1*) en topoisomerase II alpha (*TOP2A*). Alle drie de genen hebben ook prognostische waarde in BST's van mensen. PTTG1 zou theoretisch gezien een behandelingsdoelwit kunnen zijn, maar dit is nog vrij speculatief. TOP2A is echter een zeer interessant behandelingsdoelwit, omdat het meest effectieve chemotherapie protocol in mensen al remmers van topoisomerase II (dus ook TOP2A) remmers bevat. Dit zou dus ook een goede behandelingsstrategie kunnen zijn in honden met BST's met hoge *TOP2A* expressie. Als laatste zou het remmen van SF-1 activiteit ook een zeer interessante behandelingsstrategie zijn.

SF-1 speelt een essentiële rol in de ontwikkeling, groei en hormoonproductie van de bijnieren. Het is niet alleen een belangrijke factor in BST's zoals we gezien hebben in **Hoofdstuk 8**, maar het is ook een essentiële factor in HAH: een van de manieren waarop ACTH zorgt voor meer cortisol productie is namelijk via het verhogen van de SF-1 acti- viteit. Het remmen van SF-1 activiteit zou daarom interessant zijn voor honden met beide varianten van het syndroom van Cushing. Gelukkig zijn er stoffen geïdentificeerd die de SF-1 activiteit kunnen remmen, genaamd SF-1 inverse agonisten. In **Hoofdstuk 6** hebben we drie SF-1 inverse agonisten getest: IsoQ A, #31 en #32. Ook voor deze studie hebben we gebruik gemaakt van gekweekte bijnierschorscellen, dit keer zowel van normale bijnieren als van BST's. Van deze drie stoffen gaf #31 de beste resultaten: in bijnierschorscellen van normale bijnieren met en zonder ACTH stimulatie remde #31 de cortisol productie en de genexpressie van genen die aangestuurd worden door SF-1. In de bijnierschorscellen van BST's gaf #31 een wisselend effect, wat waarschijnlijk te maken heeft met de wisselende *SF-1* expressie in deze tumoren. Dit houdt in dat het effect dus afhankelijk is van de *SF-1* dosis, zoals ook al beschreven in een eerdere studie.

Een bijkomend voordeel van het remmen van SF-1 activiteit zou mogelijk zijn dat het in BST's ook de celgroei remt. Helaas groeiden de cellen in onze studie al langzaam, wat het lastig maakte om een remming op celgroei aan te tonen. Toekomstige studies zouden daarom bijvoorbeeld een langere incubatietijd kunnen aanhouden,

meer BST's kunnen includeren, een andere test kunnen gebruiken om celgroei aan te tonen, en/of bijnier-specifieke groeistimulatie kunnen toevoegen. Omdat #31 effectief de cortisol productie en genexpressie van genen die aangestuurd worden door SF-1 remde zoals aangetoond in **Hoofdstuk 6**, lijkt het waarschijnlijk dat het ook SF-1 gestimuleerde celgroei zou kunnen remmen. #31 is een interessante kandidaat om verder te ontwikkelen tot een medicijn dat *in vivo* gebruikt kan worden om cortisol productie te remmen in alle vormen van het syndroom van Cushing, en mogelijk ook om tumorgroei te kunnen remmen in honden met een BST.

Science's next top model*

In dit proefschrift zijn twee soorten modellen beschreven. In **Hoofdstukken 4, 5** en **6** hebben we gebruik gemaakt van een celkweekmodel met bijnierschorscellen, dus een *in vitro* model voor de bijnier *in vivo*. Een dergelijk kweeksysteem heeft zowel voor- als nadelen. De voordelen zijn o.a. dat de cellen hormonen produceren, genexpressie hebben van o.a. steroïdogenese enzymen, en aanzienlijk reageren op stimulatie met ACTH. De cellen zijn dus functionele bijnierschorscellen, wat interessante mogelijkheden biedt voor het onderzoeken van effecten van nieuwe medicijnen, zonder dat hierbij proefdieren gebruikt hoeven te worden. De nadelen zijn o.a. dat het *in vivo* feedbacksysteem niet aanwezig is, en dat het geen informatie geeft over bijvoorbeeld of een medicijn goed wordt opgenomen en hoe snel wordt afgebroken. Zoals al impliciet in de naam is een model slechts een benadering en niet identiek aan het systeem waar het model voor staat. Ondanks dat kunnen we concluderen dat het een nuttige techniek is voor een eerste screening en een goed beginpunt om potentieel nieuwe behandelingsmogelijkheden te testen.

Het tweede soort model is de hond als model voor de mens. Hoewel we ons in dit proefschrift vooral hebben gericht op het verbeteren van de behandeling van het syndroom van Cushing bij de hond, kunnen deze resultaten deels ook geëxtrapoleerd worden ter verbetering van de behandeling bij de mens. Er zijn namelijk veel overeenkomsten binnen deze aandoening bij de hond en bij de mens, onder andere de etiologie, de klinische verschijnselen, de behandelingsmogelijkheden en de opbouw van de bijnierschors. Daarnaast komt het syndroom van Cushing ongeveer 1000 keer zo vaak voor bij de hond als bij de mens, waardoor materiaal voor onderzoek makkelijker te verkrijgen is. De studies beschreven in dit proefschrift hebben bijgedragen aan onze kennis over de normale bijnierschors en BSTe's van honden, waardoor we meer inzicht hebben gekregen in de toepasbaarheid van de hond als diermodel. Belangrijk bij het kiezen van het geschikte diermodel is weten wat je wilt onderzoeken, en of bijvoorbeeld de factor die je wilt remmen ook aanwezig is en net zo belangrijk is in dat diermodel. Voor de middelen die we in dit proefschrift getest hebben, dus AA, MC2R antagonisten en SF-1 inverse agonisten, zijn de doelwitten aanwezig en lijken ze net zo belangrijk te zijn in honden als in mensen. Honden zouden daarom nuttige

* Naam overgenomen met toestemming van Honors Program Diergeneeskunde studenten 2017-2018

diermodellen zijn om deze middelen verder te testen in *in vivo* studies, met het belangrijke bijkomende voordeel dat deze resultaten niet alleen nuttig zijn om de behandeling van het syndroom van Cushing bij mensen te verbeteren, maar ook bij honden.

Algemene conclusies

De studies in dit proefschrift hebben bijgedragen aan het voorspellen van de prognose van honden met een BST, het identificeren van doelwitten die we mogelijk medicamenteus aan zouden kunnen pakken, en het vergaren van kennis over de werking en potentie van nieuwe behandelingsmogelijkheden. Op de resultaten die in dit proefschrift zijn beschreven kan voortgeborduurd worden in toekomstige studies, met als hoofddoel het verbeteren van het aantal en de kwaliteit van beschikbare behandelingsmogelijkheden voor het syndroom van Cushing bij zowel honden als mensen. De nieuwe medicijnen die in dit proefschrift zijn getest kunnen mogelijk superieur zijn ten opzichte van de huidige behandelingsmogelijkheden, en/of een alternatief bieden voor patiënten die niet goed reageren op de huidige behandelingsopties. Daarnaast zouden meerdere medicijnen ingezet kunnen worden in een combinatie therapie, waarbij het gebruik van twee of meerdere medicijnen de effectiviteit zouden kunnen vergroten terwijl de toxiciteit wordt beperkt. Als in de toekomst meerdere medicijnen beschikbaar zijn die prognostische factoren in BST's remmen, zou de behandeling van honden met een hoog risico op recidief van de BST na operatie kunnen worden gebaseerd op het moleculaire kwaadaardigheidsprofiel van de tumor, in het kader van gepersonaliseerde behandeling.

Belangrijkste bevindingen

Hormoonproductie (remmen)

- Honden lijken maar één functioneel CYP11B enzym te hebben dat de laatste stappen in zowel cortisol als aldosteron productie katalyseert.
- CYP17A1 komt tot expressie in de zF en zR van de bijnierschors van honden, en lijkt het enige enzym te zijn dat nodig is voor de productie van cortisol maar niet van aldosteron.
- De CYP17A1 remmer AA remt de productie van cortisol maar niet van aldosteron in gekweekte bijnierschorscellen. AA remt niet alleen CYP17A1, maar in mindere mate ook CYP21A2 en CYP11B1. *In vivo* studies zijn nodig om te bepalen of AA als behandeling voor het syndroom van Cushing bij honden in kan worden gezet.
- Twee stoffen, #776 en #299, waren effectieve antagonisten van de MC2R in gekweekte bijnierschorscellen van honden, waarbij #299 het meest potent was. Meer studies zijn nodig om te bepalen of ze selectief zijn voor de MC2R ten opzichte van andere melanocortin receptoren.

Prognostische factoren (aangrijpen)

- Minder dan 25% van alle histopathologische kenmerken die we hebben geanalyseerd in BST's van honden zijn makkelijk te scoren. Van deze waren de Ki67 proliferatie index, het percentage cellen met helder/gevacuoliseerd cytoplasma en de aanwezigheid van necrose geassocieerd met een slechtere overleving van honden met een cortisol-producerende BST na operatie.
- Als we de histopathologische Utrecht score met afkapwaardes van 6 en 11 gebruikten, konden we honden indelen in drie groepen, waarbij de groepen met hogere Utrecht scores significant kortere overlevingstijden hadden dan de groepen met lagere scores.
- Van 14 kandidaat genen was de genexpressie van drie genen significant geassocieerd met overleving na operatie: *SF-1, PTTG1* en *TOP2A*. Op basis hiervan zou het nuttig kunnen zijn om honden met hoge *TOP2A* expressie na te behandelen met topoisomerase remmers. Daarnaast zou het remmen van SF-1 activiteit een interessante aanpak kunnen zijn.
- Van de drie SF-1 inverse agonisten die we hebben getest in gekweekte bijnierschorscellen van honden was #31 het meest effectief. #31 lijkt dus een interessante kandidaat voor verdere ontwikkeling in een medicijn dat *in vivo* gebruikt kan worden om cortisol productie te remmen in alle honden met het syndroom van Cushing, maar mogelijk ook om tumorgroei te remmen in honden met een BST met hoge *SF-1* expressie.

Science's next top model

- Gekweekte bijnierschorscellen van honden produceren hormonen, hebben genexpressie van steroïdogenese enzymen, en reageren aanzienlijk op ACTH stimulatie. Dit is daarom een waardevol model om de potentie van nieuwe medicijnen te evalueren als screeningsmethode, zodat *in vivo* testen alleen wordt gereserveerd voor veelbelovende medicijnen.
- Omdat CYP17A1 tot uiting komt in de bijnierschors van honden en mensen, omdat de MC2R essentieel is in HAH bij zowel honden als mensen, en omdat *SF-1, PTTG1*, en *TOP2A* prognostische waarde hebben in zowel honden als mensen, zijn honden met het syndroom van Cushing nuttige diermodellen om de potentie van remmers van deze factoren in te testen.

&

Addendum

List of Abbreviations

#31	F1808-0154
#32	F1808-0165
#229	BIM-22A299
#776	BIM-22776
AA	abiraterone acetate
ACA	adrenocortical adenoma
ACC	adrenocortical carcinoma
ACON	adrenocortical oncocytic neoplasm
ACT	adrenocortical tumor
ACTH	adrenocorticotropic hormone
Ad4BP	adrenal 4-binding protein
AGTR2	angiotensin II receptor
AVP	arginine-vasopressin
BAC	bacterial artificial chromosome
BCL2	B-cell lymphoma 2
BSA	bovine serum albumin
BST	bijnierschorstumor
cAMP	cyclic AMP
CCND1	cyclin D1
CI	confidence interval
CRH	corticotropin-releasing hormone
CS	Cushing's syndrome
CT	cycle threshold
CYB5	cytochrome b5
CYP11A1	cytochrome P450 side chain cleavage
CYP11B1	11β-hydroxylase
CYP11B2	aldosterone synthase
CYP17A1	17α-hydroxylase/17,20-lyase

CYP21A2	21-hydroxylase
D4A	Δ^4-abiraterone
DAB	disabled 2
DAX1	dosage-sensitive sex reveral-adrenal hypoplasia congenita critical region on the X chromosome gene 1
DHEA	dehydroepiandrosterone
DHEAS	dehydroepiandrosterone sulfate
DMSO	dimethyl sulfoxide
ECL	enhanced chemiluminescence
EDP-M	etoposide, doxorubicin, cisplatin - mitotane
FBS	fetal bovine serum
G10	CH82-485G10
HAH	hypofyse-afhankelijk hypercortisolisme
HDDST	high-dose dexamethasone suppression test
HPA	hypothalamic-pituitary-adrenal
HPF	high power field
HPRT	hypoxanthine-guanine phosphoribosyltransferase
HSD3B2	3β-hydroxysteroid dehydrogenase type 2
IHC	immunohistochemistry
INHA	inhibin alpha subunit
IsoQ A	SID7969543
IsoQ B	SID7970631
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LRT	low risk of recurrence tumor
M08	CH2-265M08
MC2R	melanocortin 2 receptor
mCRPC	metastatic castration-resistant prostate cancer
MHRT	moderate/high risk of recurrence tumor
MRAP	melanocortin 2 receptor accessory protein
mRNA	messenger RNA
NGS	normal goat serum
P/B	pituitary height/brain area
PBX1	pre-B-cell leukemia transcription factor 1
PDH	pituitary-dependent hypercortisolism
PI	proliferation index
POMC	pro-opiomelanocortin
PR	progesterone receptor

PTTG1	pituitary tumor-transforming gene-1
RAC1	Ras-related R3 botulinum toxin substrate 1
RIA	radioimmunoassay
RIPA	radioimmunoprecipitation
RPS5	ribosomal protein S5
RPS19	ribosomal protein s19
RRM2	ribonucleoside-diphosphate reeducate subunit M2
RT	radiotherapy
RT-qPCR	quantitative RT-PCR
SDHA	succinate dehydrogenase complex subunit A
SF-1	Steroidogenic factor-1
SOAT1	sterol-O-acyltransferase 1
SRPR	small proline-rich protein
SRS	stereotactic radiosurgery
StAR	steroidogenic acute regulatory protein
TBS	Tris buffered saline
TBST0.1%	Tris buffered saline with 0.1% tween
TOP2A	topoisomerase II alpha
UCCR	urinary corticoid:creatinine ratio
VAV2	Vav Guanine Nucleotide Exchange Factor 2
VNN1	pantetheinase
Wnt4	Wingless-type MMTV integration site family, member 4
YWHAZ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta
zF	zona fasciculata
zG	zona glomerulosa
zR	zona reticularis

Acknowledgments/Dankwoord

Als klein meisje had ik twee grote dromen: ik wilde dierenarts worden en ik wilde een boek schrijven. Hoewel ik toen een wat ander beeld voor ogen had, kan ik nu toch zeggen dat beide dromen zijn uitgekomen. Uiteraard komt er bij het verrichten van onderzoek en het schrijven van een proefschrift erg veel kijken, en had ik dit nooit in mijn eentje voor elkaar kunnen krijgen. Ik wil heel veel mensen daarom hartelijk bedanken, waarvan een aantal in het bijzonder.

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Promotoren
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Curriculum Vitae

Karin Sanders was born on February 9, 1990 in Nuenen, The Netherlands. After graduating from her high school Augustinianum in Eindhoven in 2008, she started to study Veterinary Medicine at the Faculty of Veterinary Medicine, Utrecht University.

In the Bachelor program her interest for the subject endocrinology began to grow, which is why she decided to write her Bachelor thesis on the subject of adrenal cortex organogenesis and the role of stem/progenitor cells under supervision of Dr. Hans S. Kooistra, for which she won the Honorable Mention award in 2011. She started her Master Companion Animal Medicine in 2011, and because her interest in endocrinology and the adrenal cortex had only increased, the subject of her research internship was the functional zonation of the canine adrenal cortex, under supervision of Dr. Sara Galac and Dr. Jan A. Mol. Although this internship took only four months, it was such a positive experience that it had initiated a great motivation in doing research. Therefore, when her supervisor Dr. Sara Galac mentioned that they would like to have another PhD candidate on the subject, Karin jumped at the opportunity. After graduating as a veterinarian at the end of 2014, she started to work on her PhD project at the beginning of 2015. During her PhD track Karin has presented her work on multiple national and international conferences. She has won multiple awards with her work, including the Oxford Laboratories Award of the Society for Comparative Endocrinology for the best article in the category small animal endocrinology in 2016; the award for the best presentation of the European Society of Veterinary Endocrinology at the ECVIM-CA congress 2017 in St. Julian's, Malta; and the George Fleming Prize for the most meritorious paper published in The Veterinary Journal in 2018.

Karin wants to pursue her career in research and is currently doing so as a postdoctoral researcher at the Faculty of Veterinary Medicine, Utrecht University. Together with her partner Robbert and their two cats Zola and Bailey, Karin currently lives in Zeist, The Netherlands.

Karin Sanders is geboren op 9 februari 1990 in Nuenen, Nederland. Na het behalen van haar VWO-diploma aan het Augustinianum te Eindhoven in 2008, begon ze met de studie Diergeneeskunde aan de Faculteit Diergeneeskunde, Universiteit Utrecht.

Tijdens de Bachelor schreef ze haar scriptie over de ontwikkeling van de bijnierschors en de rol van stam-/progenitor cellen daarbij, onder begeleiding van dr. Hans S. Kooistra, waarvoor ze de Eervolle Vermelding prijs won in 2011. Ze begon in 2011 met haar Master Geneeskunde van Gezelschapsdieren, en omdat haar interesse in de endocrinologie ondertussen nog verder was gegroeid, verdiepte ze zich tijdens haar onderzoekstage in de functionele zonering van de bijnierschors van honden, onder begeleiding van dr. Sara Galac en dr. ir. Jan A Mol. Ondanks dat deze stage slechts vier maanden duurde, was het een dergelijk positieve ervaring dat het een grote motivatie voor het doen van onderzoek had geïnitieerd. Toen haar begeleidster dr. Sara Galac liet vallen dat ze graag nog een PhD kandidaat op het onderwerp wilden hebben, greep Karin die kans met beide handen aan. Nadat ze eind 2014 afstudeerde als dierenarts is ze begin 2015 begonnen met haar promotieonderzoek. Tijdens haar promotietraject heeft Karin haar werk gepresenteerd op verschillende nationale en internationale congressen. Met haar werk heeft ze verschillende prijzen gewonnen, waaronder de Oxford Laboratories Award van de Society for Comparative Endocrinology voor het beste artikel in de categorie fundamentele wetenschap van gezelschapsdieren in 2016; de prijs voor de beste presentatie van de European Society of Veterinary Endocrinology op het ECVIM-CA congres 2017 in St. Julians, Malta; en de George Fleming prijs voor het meest verdienstelijke artikel in The Veterinary Journal in 2018.

Karin wil graag haar carrière in het onderzoek voortzetten en doet dit momenteel als postdoctoraal onderzoekster aan de Faculteit Diergeneeskunde, Universiteit Utrecht. Samen met haar partner Robbert en hun twee katten Zola en Bailey woont Karin momenteel in Zeist, Nederland.

List of Publications

Sanders K, Cirkel K, Grinwis GCM, Teske E, Van Nimwegen SA, Mol JA, Hesselink JW, Kooistra HS, Galac S. The Utrecht score: a novel histopathological scoring system to assess the prognosis of dogs with cortisol-secreting adrenocortical tumors. *Veterinary and Comparative Oncology, Accepted*. https://doi.org/10.1111/vco.12474.

Sanders K, De Wit WL, Mol JA, Kurlbaum M, Kendl S, Kroiss M, Kooistra HS, Galac S. Abiraterone acetate for Cushing's syndrome: study in a canine primary adrenocortical cell culture model. *Endocrinology* (2018) 159(11);3689-3698. https://doi.org/ 10.1210/en.2018-00588.

Sanders K, Kooistra HS, Galac S. Treating canine Cushing's syndrome: current options and future prospects. *The Veterinary Journal* (2018) 241;42-51. https://doi.org/ 10.1016/j.tvjl.2018.09.014. (*Awarded the George Fleming Prize for the most meritorious paper published in The Veterinary Journal in 2018*)

Sanders K, Mol JA, Kooistra HS, Galac S. Melanocortin 2 receptor antagonist in canine pituitary-dependent hypercortisolism: *in vitro* studies. *Veterinary Research Communications* (2018) 42(4);283-288. https://doi.org/10.1007/s11259-018-9737-x.

Pijnacker T, Knies M, Galac S, **Sanders K**, Mol JA, Kooistra HS. TRH-induced secretion of adrenocorticotropin and cortisol in dogs with pituitary-dependent hypercortisolism. *Veterinary Quarterly* (2018) 38(1);72-78. https://doi.org/ 10.1080/01652176.2018.1521537.

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. *Domestic Animal Endocrinology* (2018) 63;23-30. https://doi.org/10.1016/j.domaniend.2017.11.001.

Sanders K, Mol JA, Kooistra HS, Slob A, Galac S. New insights in the functional zonation of the canine adrenal cortex. *Journal of Veterinary Internal Medicine* (2016) 30(3);741-750. https://doi.org/10.1111/jvim.13946. *(Awarded the Oxford Laboratories Award for best publication in the field of small animal endocrinology in 2016)*

Manuscripts in preparation

Sanders K, Van Staalduinen GJ, Uijens MCM, Mol JA, Teske E, Slob A, Hesselink JW, Kooistra HS, Galac S. Molecular markers of malignancy in canine cortisol-secreting adrenocortical tumors. *Submitted.*