Pathogenesis of canine cortisol-secreting adrenocortical tumors

Pathogenese van cortisol-producerende bijniertumoren bij de hond

(met een samenvatting in het Nederlands)

Proefschrift

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Chapter 1

Aims and scope of the thesis

Hypercortisolism or Cushing's syndrome is one of the most common endocrine disorders in dogs, and is caused by a cortisol-secreting adrenocortical tumor (AT) in approximately 15-20% of the cases. Cortisol-secreting ATs are characterized by autonomous or ACTH-independent hypersecretion of cortisol and uncontrolled tumor growth, the molecular mechanisms of which are largely unknown. Treatment options in dogs with a cortisol-secreting AT are limited. The preferred treatment consists of complete adrenalectomy, provided no visible metastases or ingrowth in blood vessels are present. When adrenalectomy is not possible or in case of recurrence, mitotane monotherapy is the treatment of choice. Alternatively, palliative treatment using a competitive inhibitor of 3β-hydroxysteroid dehydrogenase can be used to decrease cortisol secretion and control the clinical signs of hypercortisolism. For the development of new targeted treatments it is essential to know which cellular pathways play a key role in adrenocortical tumorigenesis. It may very well be that elucidating the pathways that play a role in the pathogenesis of canine ATs can also be translated to human medicine. Consequently, the much higher incidence of cortisol-secreting ATs in dogs when compared to humans, opens the possibility that the dog might be a good spontaneous, large animal model of human adrenocortical carcinoma (ACC).

The aims of this thesis were:

a) To investigate molecular pathways, of which alterations may lead to autonomous cortisol secretion and tumor growth in canine cortisol-secreting ATs
b) To search for potential therapeutic targets and prognostic markers in canine cortisol-secreting ATs
c) To evaluate the value of dogs with a spontaneous cortisol-secreting AT as a model for human ACC

The general introduction (**Chapter 2**) provides an overview of the comparative adrenal morphology and function in dogs and humans, followed by a description of the characteristics and current treatment options for canine and human cortisol-secreting ATs. Subsequently, the characteristics of comparative oncology in pet dogs are addressed, along with the potential advantages for the research and treatment of cortisol-secreting ATs in both dogs and humans.

One of the main characteristics of cortisol-secreting ATs is the presence of cortisol hypersecretion in the absence of ACTH stimulation. Previous research has shown that this phenomenon cannot be explained by a rise in ACTH receptor (*MC2R*) or

steroidogenic enzyme expression. In human cortisol-secreting ATs, alterations in the intracellular MC2R/cyclic AMP (cAMP)/protein kinase A (PKA) signaling pathway, the pathway that leads to cortisol secretion, occur frequently. The aim of the study described in **Chapter 3** was to investigate alterations in this pathway in 44 canine cortisol-secreting ATs by means of mutation analysis of the genes encoding *MC2R*, G-protein stimulatory subunit α (*GNAS*) and protein kinase A regulatory subunit 1 A (*PRKAR1A*).

Activation of cAMP signaling may also be due to altered hormone receptor expression, which may allow the use of hormone receptor agonists or antagonists, i.e., which may provide a unique opportunity for targeted treatment. In human ATs, altered expression of progesterone receptor (*PR*), growth hormone receptor (*GHR*), dopamine receptors (*DRs*) and somatostatin receptors (*SSTRs*) has been reported. In the study described in **Chapter 4** the presence and relative mRNA expression of *PR*, *GHR*, *SSTR1-5* and DRD_1-D_5 were investigated in canine cortisol-secreting adenomas and carcinomas, and the results were related to clinical outcome.

A key mediator of MC2R/cAMP-induced cortisol synthesis is steroidogenic factor 1 (SF-1). Recent studies in humans and mice have also indicated that increased expression of *SF-1* is an important factor in adrenocortical tumorigenesis. In the study described in **Chapter 5** the relative mRNA expression of *SF-1* was assessed in 36 canine ATs and 10 normal adrenal glands. Additionally, SF-1 protein expression was evaluated by means of immunohistochemistry, and the results were related to steroidogenesis and clinical outcome.

An important downstream target of cAMP/SF-1 signaling is the expression of the angiogenic gene angiopoietin 2 (*ANGPT2*). Angiogenesis, the process of new blood vessel formation from preexisting vasculature, is a major factor in tumor development. By inducing angiogenesis, a tumor may provide itself with the nutrients and oxygen necessary for growth. In addition, angiogenesis may facilitate distant metastasis. Consequently, the development of drugs targeting angiogenesis is a hot topic in human cancer treatment. In the study described in **Chapter 6** the role of angiogenesis in canine ATs was addressed. In this study, the relative mRNA expression of the genes encoding *ANGPT1* and *2*, their receptor *Tie2*, vascular endothelial growth factor (*VEGF*), its receptors (*VEGFR1* and *2*) and basic fibroblast growth factor (*bFGF*) was assessed. Furthermore, the protein expression of ANGPT2 was evaluated by means of western blotting and ANGPT2 and Tie2 localization were evaluated immunohistochemically. Finally, the regulation of *ANGPT2* expression was addressed using the human H295R adrenocortical carcinoma cell line.

Another potential target of cAMP signaling is the canonical Wnt pathway. Activation of this pathway induces cell proliferation and survival. Activation of the canonical Wnt pathway can be caused by activating mutations in the genes encoding β-catenin (*CTNNB1*) or *AXIN2*, increased Wnt-ligand expression or decreased Wnt-inhibitor expression. Activation of the Wnt pathway is a frequent event in human ATs and has been linked to a poor prognosis. In the study described in **Chapter 7** the role of the Wnt pathway in canine ATs is addressed. Activation of the Wnt-pathway was assessed using immunohistochemical staining for β-catenin and mRNA expression analysis of Wnt-target genes. The cause of activation was assessed by means of mutation analysis of *CTNNB1* and *AXIN2*, and mRNA expression analysis of Wnt-ligands and Wnt-inhibitors. The results were related to clinical outcome and *GNAS* mutations status.

Like canonical Wnt signaling, phosphatidylinositol 3 kinase (PI3K) – AKT signaling induces cell proliferation and reduces apoptosis. Consequently, several inhibitors of this pathway have been developed for cancer treatment. In human ATs, PI3K activation is frequently detected and may be caused by increased expression of insulin-like growth factor 2 (*IGF-II*) and the type 1 IGF receptor (*IGFR1*). In the study described in **Chapter 8** the role of the PI3K pathway in canine ATs is addressed. Activation of this pathway was assessed by means of mRNA expression analysis of PI3K target genes. The cause of activation was evaluated by means of mutation analysis of phosphatase and tensin homolog (*PTEN*) and PI3K catalytic subunit A (*PIK3CA*), and mRNA expression analysis of *IGF-I* and *IGF-II*, IGF receptors, endothelial growth factor (EGF) receptors and *PTEN*. The results were related to clinical outcome.

In **Chapter 9**, the outcome of the studies investigating the molecular pathogenesis of autonomous growth and cortisol secretion, therapeutic targets and prognostic markers in canine ATs, and their implications for the treatment of dogs with a cortisol-secreting AT are summarized and discussed. Additionally, our results are compared with the present knowledge on these topics in human ACC and placed in the context of the suitability of the dog as a spontaneous model for human ACC.

Chapter 2

General introduction

One of the main focuses of present day cancer research is the concept of personalized medicine, in which not just the tumor type, but more importantly the molecular alterations in cancer-related signaling pathways determine treatment. In the past decade important progress has been made in determining the contributing factors and signaling pathways for different types of cancer, and implementing this knowledge in the development of new treatment modalities. Comparative oncology, by using a reliable and homologous animal model, can facilitate this type of research and be helpful in determining the role of molecular alterations in carcinogenesis and in translating fundamental research into the development of new therapeutic modalities. In this introduction we address the value of new approaches for the treatment of adrenocortical tumors in dogs and the value of the dog as a model for human adrenocortical carcinoma (ACC).

Human ACC is a rare, but highly malignant disease. The lack of efficient treatment for advanced or metastatic disease results in a very poor prognosis, in particular in case of cortisol-secreting ACC [1]. Research into the molecular origin of cortisol-secreting ACC and the development of new treatments is complicated by the low availability of human tumor material and normal control tissue. Mouse models are frequently used, but have a number of disadvantages, such as the differences in adrenal morphology, the absence of spontaneously arising ACC, the lack of tumor heterogeneity and the absence of environmental factors influencing carcinogenesis. Domestic ferrets have also been used as a model for human ACC, as they develop gonadectomy-induced functional adrenocortical tumors (ATs) [2]. However, these ATs secrete typically androgens rather than glucocorticoids. Dogs are genetically and anatomically more similar to humans than mice or ferrets, develop cortisol-secreting ATs spontaneously and share the human living environment [2]. Consequently, pet dogs may be a much better spontaneous (large) animal model than mice for human ACC. In the following paragraphs, the comparative adrenal morphology and function, AT characteristics and current ACC treatment options in humans and dogs will be discussed, followed by a discussion of the characteristics of comparative oncology in pet dogs and its potential advantages for the research and treatment of cortisol-secreting ATs in both humans and dogs.

Comparative adrenal morphology and function

With regard to the anatomy and physiology of the adrenal cortex, humans and dogs show a high level of homology [2]. In both species, the bilateral adrenal glands are situated craniomedial to the kidneys, and consist of an inner medulla and an outer cortex, surrounded by a fibrous capsule. In the adrenal cortex, cytochrome P-450

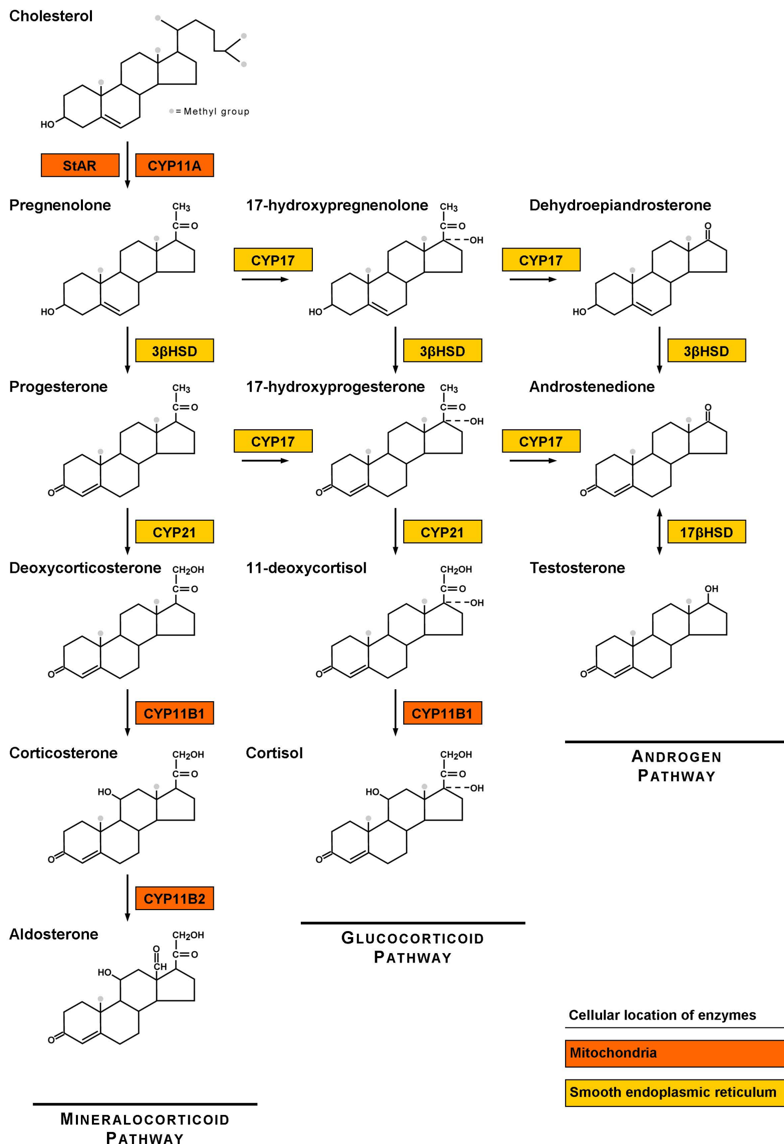

Figure 1

Schematic representation of steroidogenesis in the adrenal cortex.

Upon ACTH stimulation, steroidogenic acute regulatory protein (StAR) facilitates transport of cholesterol to the inner mitochondrial membrane, where it is converted to pregnenolone by cytochrome P450 cholesterol side chain cleavage (CYP11A). Subsequently, pregnenolone is converted through multiple enzyme steps to either aldosterone (mineralocorticoid pathway), cortisol (glucocorticoid pathway) or testosterone (androgen pathway).

Abbreviations: HSD3B: 3β-hydroxysteroid dehydrogenase, CYP17: 17β-hydroxylase/17,20-lyase, CYP21: 21-hydroxylase, CYP11B1: 11β-hydroxylase type 1, 17βHSD: 17β-hydroxysteroid dehydrogenase Illustration from: Galac S, Reusch CE, Kooistra HS, Rijnberk A. Adrenals. In: Clinical endocrinology of dogs and cats, Hannover: Schlütersche; 2010, p. 95

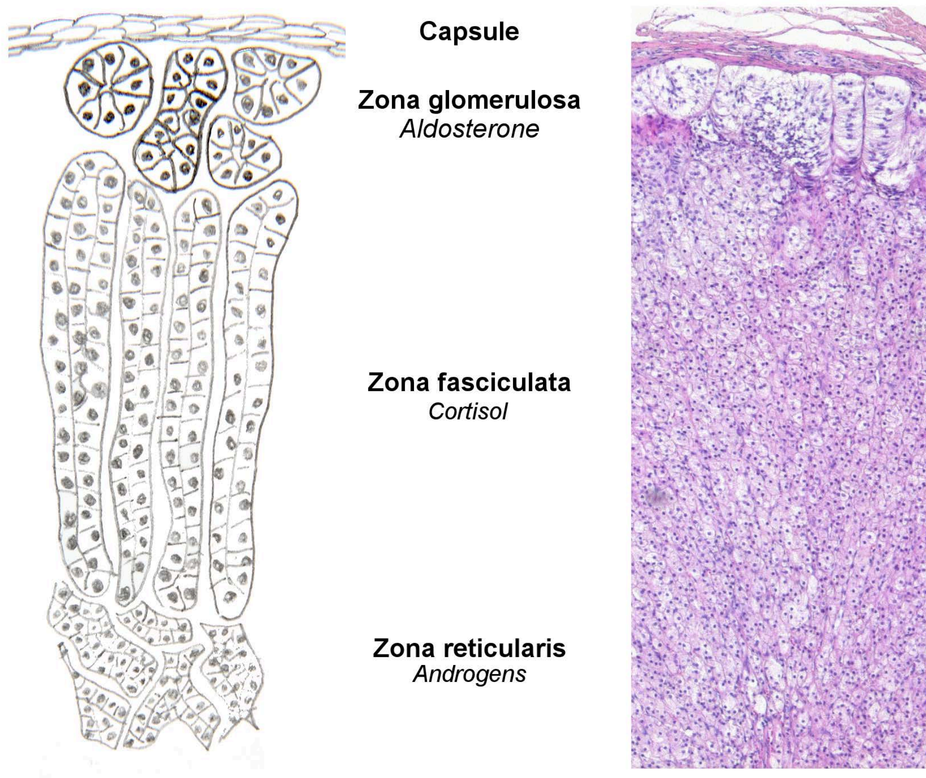

Figure 2
Zonation of the adrenal cortex.
Schematic illustration of the functional zonation of the adrenal gland (A), showing in cross-section the medulla and the cortex, which is subdivided into three functional zones. The subcapsular zona glomerulosa (ZG) produces mainly mineralocorticoids, the middle zona fasciculata (ZF) is the main source of cortisol secretion and the inner zona reticularis (ZR) produces mainly androgens. Haematoxylin and eosin (HE) stained section of the adrenal cortex (B), showing the capsule and the three cortical zones.

enzymes are responsible for most of the enzymatic conversions from cholesterol to mineralocorticoids, glucocorticoids and androgens (Fig. 1) [3,4].

Three functional and morphological layers can be distinguished in the adrenal cortex: the outermost zona glomerulosa (ZG), the middle zona fasciculata (ZF), and the inner zona reticularis (ZR). The latter two zones function together as a unit, and are the main source of the body's glucocorticoid secretion (Fig. 2) [3]. In the healthy individual, corticosteroid production and secretion is strictly regulated by the hypothalamic-pituitary-adrenocortical axis (HPA axis). In response to stress, episodic or immune-mediated signals, corticotropin-releasing hormone (CRH) and arginine-vasopressin (AVP) from the hypothalamus induce the synthesis of adrenocorticotropic hormone (ACTH) from proopiomelanocortin (POMC) in the

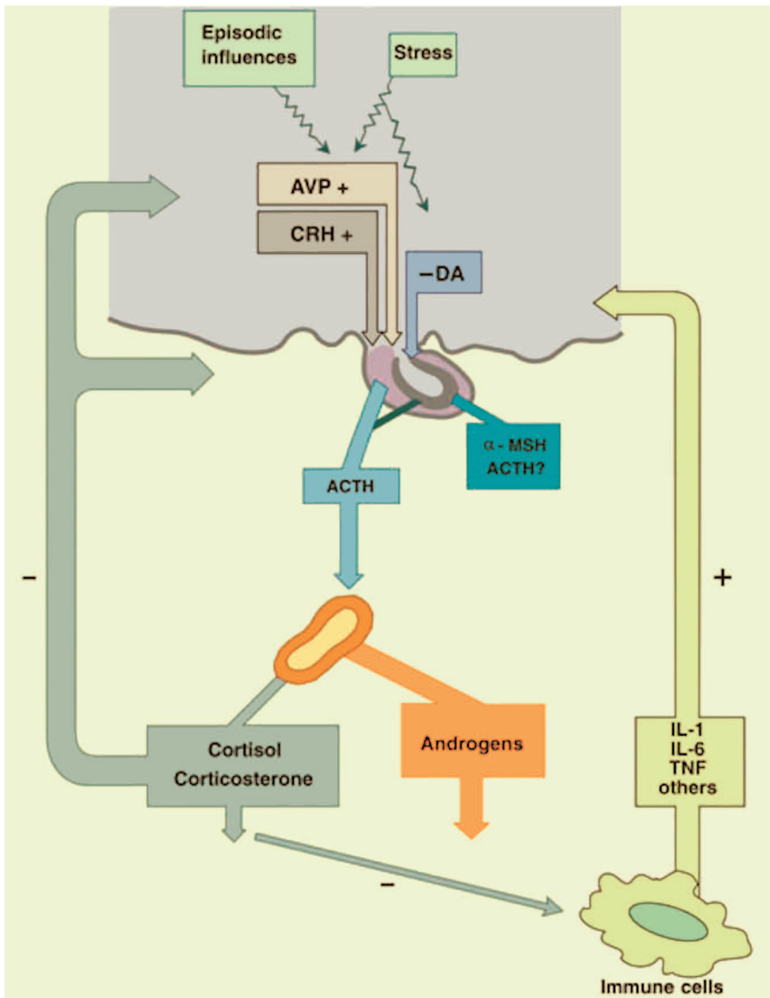

Figure 3

Schematic illustration of cortisol regulation by the hypothalamic – pituitary – adrenocortical (HPA) axis. In response to stress, episodic or immune-mediated signals, corticotropin-releasing hormone (CRH) and arginine-vasopressin (AVP) from the hypothalamus induce adrenocorticotropic hormone (ACTH) production and release in the anterior lobe of the pituitary gland. In the adrenal cortex, ACTH stimulates the production and secretion of cortisol. By a negative feedback mechanism, plasma cortisol inhibits both CRH and ACTH release, thus creating a negative feedback loop that controls cortisol levels.

Illustration from: Galac S, Reusch CE, Kooistra HS, Rijnberk A. Adrenals. In: Clinical endocrinology of dogs and cats, Hannover: Schlütersche; 2010, p. 98

anterior lobe of the pituitary gland (Fig. 3) [3]. After its release into the circulation, ACTH binds to its receptor on the adrenocortical cells, the G-protein coupled melanocortin 2 receptor (MC2R), initiating the intracellular signaling cascade that leads to cortisol secretion (Fig. 4) [3]. Upon binding of ACTH, the G protein

stimulatory subunit alpha (Gs-alpha) is converted to its active, guanidine triphosphate (GTP)-bound, configuration, and in turn activates adenylyl cyclase to convert ATP into cAMP [5]. Next, cAMP serves to activate protein kinase A (PKA), which activates several transcription factors and steroidogenic acute regulatory protein (StAR). The latter mediates the immediate response to an ACTH stimulus, by facilitating the transport of cholesterol to the inner mitochondrial membrane, where steroidogenesis takes place [6]. The transcription factors serve to induce the transcription of genes encoding the enzymes which are involved in steroidogenesis. As cortisol cannot be stored in the adrenocortical cells, it is secreted directly after synthesis into the circulation. The relocation of cholesterol and enzyme expression levels determine the rate of cortisol release into the circulation [3].

Following cortisol release, a negative feedback loop ensures the normalization of cortisol concentrations and their return to the baseline level. This negative feedback is initiated by binding of cortisol to glucocorticoid receptors (GR) and mineralocorticoid receptors (MR), and results in inhibition of the ACTH-releasing factors CRH and AVP from the hypothalamus as well as of ACTH from the anterior lobe of the pituitary, and thus inhibits the production of cortisol [3]. Changes in GR and MR expression occur during aging in the dog and may thereby contribute to increased cortisol secretion during the aging process [7].

Clinical characteristics of cortisol-secreting ATs

Cortisol-secreting ATs occur in both humans and dogs, and disturb the strict regulation of cortisol secretion. In dogs, the incidence of cortisol-secreting ATs is a 1000-fold higher than in humans: hypercortisolism occurs in 1 to 2 cases per 1000 dogs per year, and approximately 15% of these cases is due to cortisol-secreting adrenocortical adenomas or carcinomas [3]. A correct distinction between adenomas and carcinomas based on histological evaluation can sometimes be difficult in dogs. According to the criteria described by Labelle *et al.*, the histological characteristics of adrenocortical carcinomas in dogs include evidence of vascular invasion, peripheral fibrosis, capsular invasion, trabecular growth, hemorrhage, necrosis, and single cell necrosis. Typical histological characteristics of adenomas were hematopoiesis, fibrin thrombi, and cytoplasmic vacuolization [8]. Based on these criteria, the majority of ATs in dogs are classified as carcinomas. Cortisol-secreting ATs occur mostly in middle-aged to elderly dogs, without breed or sex predilection [3]. Dogs typically present with clinical signs due to hypercortisolism, which are largely comparable to those in humans, and include centripetal obesity, lethargy, exercise intolerance,

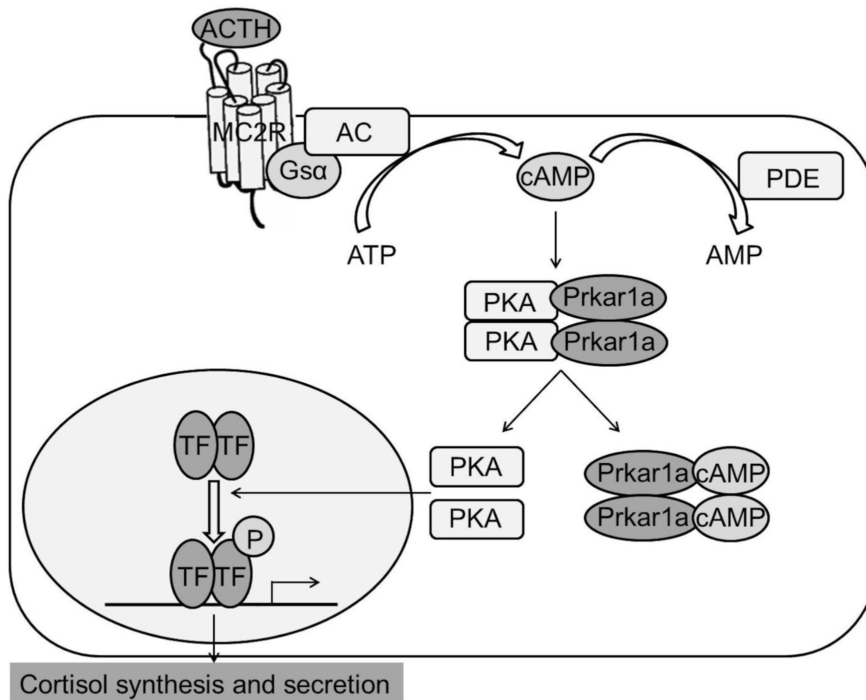

Figure 4
Schematic overview of the MC2R-cAMP-PKA pathway.
Binding of ACTH to the MC2R activates adenylate cyclase (AC) through Gsα. AC converts ATP to cAMP, which separates protein kinase A (PKA) from its regulatory subunit PRKAR1A, enabling it to phosphorylate (P) and subsequently activate transcription factors (TF). Phosphodiesterase (PDE) inhibits the pathway by converting the active cAMP to inactive AMP.
Abbreviations: MC2R: melanocortin 2 receptor, Gsα: stimulatory G protein alpha subunit, ATP: adenosine triphosphate, cAMP: cyclic adenosine monophosphate, AMP: adenosine monophosphate, PRKAR1A: protein kinase A regulatory subunit 1A

muscle weakness and atrophy, and skin atrophy. Additionally, dogs commonly display hair loss, polyphagia, polyuria and polydipsia. Complications include secondary diabetes mellitus, systemic hypertension, pulmonary thromboembolism, and congestive heart failure [3].

In humans, sporadic adrenocortical carcinoma (ACC) is a rare, but extremely malignant form of cancer [1]. The incidence is estimated at 0.5 to 2 cases per million people per year, of which approximately one-third is cortisol secreting. Women are more frequently affected than men (ratio 1.5) and the average age of onset is around 40-50 yr. [9]. According to the criteria developed by the European Network for the Study of Adrenal Tumors, sporadic ACC can be classified into 4 stages of disease according to size, localization and the presence of metastases. Stage 1 (tumor size < 5cm) and stage 2 (tumor size > 5cm) represent localized disease, while in stage 3

tumor invasion into local surrounding tissues or lymph nodes is present, and stage 4 is characterized by the presence of distant metastases [10]. Typically, at the time of diagnosis, patients are in advanced stages of disease, with large tumors and distant metastases being present in approximately 25-50% of cases [1]. Lung, liver and bone are the most common metastatic sites [1]. The clinical signs can be divided into signs due to tumor growth, such as abdominal or flank pain and a distended abdomen, and signs due to hormone excess. In patients primarily presenting with signs due to hormone excess, signs of rapidly progressing hypercortisolism or Cushing's syndrome are the most common [1]. These include centripetal fat deposition, muscle weakness and atrophy, lethargy and exercise intolerance, thinning of the skin, delayed wound healing, osteoporosis, secondary diabetes mellitus, hypokalemia and hypertension [11].

Treatment and prognosis of cortisol-secreting ATs

In dogs, the preferred treatment for localized ATs consists of complete adrenalectomy [12], with the laparoscopic approach becoming the preferred method [13]. No adjuvant treatment is offered in dogs, and although surgery may be complicated by the presence of ingrowth in the vena cava, complete resection of the AT is often curative. The median postoperative survival time has been reported at 48 months [14]. However, recurrent signs of hypercortisolism after adrenalectomy were reported in 8 of 27 dogs with ACC, due to either regrowth of the tumor or distant metastasis [14]. In these cases, and in dogs with evidence of distant metastasis at presentation, mitotane (Lysodren®) monotherapy is the treatment of choice [15]. Alternatively, palliative treatment using a competitive inhibitor of 3β-hydroxysteroid dehydrogenase (3β-HSD) can be used to decrease cortisol secretion and control the clinical signs of hypercortisolism. The use of other chemotherapeutic agents for dogs with metastatic ACC has not been described, and no trials for targeted treatment have been performed yet.

In humans, the preferred treatment for ACC depends largely on tumor stage and the presence or absence of metastases [1]. In case of a resectable tumor, complete surgical removal combined with lymph node resection offers the best prospective [16]. Retrospective studies suggest that supplementing adrenalectomy with the adrenocorticolytic drug mitotane offers significant improvement of overall and disease-free survival [17]. However, even in cases of seemingly complete resection, tumor recurrence or metastatic disease occurs in a high percentage of patients [9]. When evidence of metastatic disease is already clear at initial presentation, complete surgical removal of tumor tissue is no longer feasible [18]. These patients may be

treated with a combination of mitotane, etoposide, doxorubicin and cisplatin (EDP-M), based on the results of the FIRM-ACT (First International Randomized trial in locally advanced and Metastatic Adrenocortical Carcinoma Treatment) trial [19].

Despite these treatments the prognosis of ACC in humans is very poor, with an average 5 year survival of 16-44% [20]. In patients with advanced or metastatic ACC, current treatment protocols are generally not sufficient, which has led to a number of studies investigating the potential of targeted therapy. Early phase clinical trials have been conducted, using inhibitors of the epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF) and its receptors, or treatment with sunitinib which inhibits cellular signaling by targeting multiple receptor kinases related to VEGFR signaling or platelet-derived growth factor receptors (PDGFR) [19]. Although sunitinib has resulted in modest anti-tumor effects in a phase II clinical trial [21], the results of these first trials have generally been disappointing [19]. Recent efforts have been aimed at inhibiting insulin-like growth factor (IGF) signaling, based on the observation of strong overexpression of IGF-II and the IGF type I receptor (IGFR1) in the majority of human ACCs [22]. Different strategies have included using monoclonal IGF-II antibodies (cixutumumab), small molecule IGFR1 inhibitors (OSI-906) or inhibitors of the downstream target mammalian target of rapamycin (mTOR). Stable disease in a subset of patients has been reported as the best response [19], and a large phase III trial with OSI-906 (GALACCTIC, NCT00924989) is ongoing.

Current animal models for ACC

From the previous discussion it becomes clear that the current therapeutic options for both human and canine cortisol-secreting ATs are, in many of the cases, insufficient. To facilitate the research on new, targeted treatment options, the availability of a reliable and homologous animal model would be highly advantageous. The mouse has long been the classic model for the research of human cancer, including ACC. Mouse models have been used for a multitude of purposes and the advantages of the mouse as a model system are clear: mice are small, easy to handle and maintain, have a short gestation period and fast disease progression [23]. Many different inbred strains exist, which guarantee a known and controlled genetic makeup. Furthermore, it is possible to selectively "knock out" or "knock in" genes of interest, creating unique models, for instance to illuminate the function of genes in carcinogenesis [24]. However, in spite of these advantages, the results of mouse studies often cannot be extrapolated to the human clinic, and new therapeutic modalities that were efficacious in mouse studies regularly fail in human clinical

trials [25,26]. Possible reasons for this include differences in anatomy, physiology and genetic makeup between humans and mice, as well as the limited representation of some of the crucial features of human cancer in their respective mouse models [23,25]. With regard to the adrenal cortex and adrenocortical tumorigenesis, three major differences may be noticed between humans and mice. First, in humans the adrenal cortex can be divided into three zones, one of which, the zona reticularis is absent in the adult mouse [2]. Second, in humans cortisol is the main glucocorticoid produced in the adrenal cortex, while adrenocortical cells of the mouse lack 17-alpha-hydroxylase (CYP17) expression, and consequently corticosterone is the main glucocorticoid produced [2]. Finally, and most importantly, ATs in mice do not occur spontaneously, like in humans [2]. Thus in order to study adrenocortical tumorigenesis or test drug efficacy in mice, either inducing tumor growth or the introduction of a tumor xenograft is necessary, further reducing the homology between the mouse model and human ACC.

Comparative oncology: spontaneous tumors in dogs as a model for human cancer

The above-mentioned difficulties with mouse models could be overcome by using a model organism with higher homology in adrenal function and morphology, and with a high sporadic incidence of cortisol-secreting ACC. Based on these criteria, the dog might represent a good spontaneous large animal model organism. The use of domestic dogs with spontaneous tumors as a model for human cancer has been a much discussed topic recently. Most of the cancer types that occur in humans, also occur spontaneously in dogs [23], and share many features with their human counterparts, including clinical and histological presentation, biological behavior, therapeutic response and tumor heterogeneity [23,25]. As dogs share the living environment of humans, they are subject partly to the same carcinogens and the incidence of cancer in dogs is similar to that in humans [25]. In the US alone, an estimated 73 million dogs are kept as pets, and over 1 million dogs per year are diagnosed with cancer [25]. Many pet dogs are considered members of the family by their owners. Consequently, owners are often highly motivated to seek out the best treatment for their pets and in the US over 40 billion per year is spent on dog health care [23]. Many of the drugs and treatment types used are the same as in human health care, and when conventional treatment is not available or does not suffice, the use of experimental drugs to treat canine cancer can be an option for motivated owners [23].

Unlike in humans, there are no strict regulations guiding the use of new drugs in phase I/II/III trials, and the use of such a drug is left to the discretion of the owner [27]. This allows the use of new drugs in a treatment naive population, whereas human clinical trials are often carried out on heavily pretreated patients with advanced disease. Another advantage lies in the fact that dogs age approximately 5 to 7-fold faster than humans, and disease progression is faster, while euthanasia is possible and accepted to prevent prolonged suffering [23]. This allows a shorter time span to trial end points. For instance the disease-free intervals in canine osteosarcoma and lymphoma can be assessed in 18 months, while in humans >7 years would be needed to evaluate the response rate and long term effectiveness of drugs in these cancer types [25]. The extrapolation of results to the human clinic is facilitated by the fact that pharmacokinetics and -dynamics are highly comparable between both species. In this way not only safety, but also efficacy, biomarkers, endpoints and adverse effects of a drug can be evaluated in dogs with a tumor, providing valuable information for the early phase human clinical trials and saving precious amounts of time and money [23].

Already, dogs with sporadic cancer have proven their value as a model for human cancer treatment in several ways. Early clinical trials for limb-sparing techniques in dogs with osteosarcoma have preceded the successful use of these techniques in humans [28,29]. The same holds true for intensity-modulated radiation therapy, the use of which is now widespread in human patients, in particular with head and neck cancer [30,31]. Likewise, for various other new anticancer strategies, clinical trials in dogs have preceded the first successful clinical trials in humans. Examples include the development of inhaled cytotoxic chemotherapy for lung cancer [27,32,33] and the use of Bruton tyrosinase inhibitors in spontaneous non-Hodgkin B-cell lymphoma [34]. In gene therapy, human agent delivery is mimicked more closely in dogs than in mice, due to the similarities in anatomy and size [35]. Consequently, dogs with a tumor play an important role in the development of gene therapy, and the first results have shown great promise, for instance using adeno-associated virus phage vectors (AAVP) for the targeted delivery of genes encoding tumor necrosis factor α (TNFα) [36].

Dogs with cancer may not only be of use in drug development, but also in unraveling the molecular genetic background of cancers. Next to humans, pet dogs have the highest phenotypic and genetic variability [37]. Due to the evolutionary history of dogs and their selective breeding, nowadays domestic dogs contain approximately 400 isolated populations in the form of breeds [23]. The genetic and disease heterogeneity within breeds is strongly reduced and certain breeds are predisposed to certain types of cancer. Therefore the entire dog population represents a highly

outbred population, with a huge genetic diversity, like the human population, while specific breeds may represent good models for individuals or families genetically predisposed to certain cancer types [23]. The much higher linkage disequilibrium within breeds makes dogs powerful subjects for genetic mapping [38]. This is further facilitated by the fact that the dog genome has been sequenced and a fairly accurate version of the genome assembly is available [37]. Different studies have shown that domestic dogs share a much higher homology in protein and DNA sequence with humans than mice [37]. For some canine cancer types, mutations have been found that are homologous to those in the corresponding human disease. This includes for instance mutations in known oncogenes, such as P53 [39]. Many of the candidate genes and pathways involved in human osteosarcoma, have also been implicated for osteosarcoma in dogs [40-42] and a recent study in colorectal cancer suggests that comparing genetic alterations between human and canine cancer may provide a novel strategy for distinguishing driver and passenger mutations [43].

In other cases, similar genetic alterations or pathways have been shown to lead to different tumor types in humans and dogs [25]. Mutations in the cKIT oncogene cause gastrointestinal stromal tumors in humans [44] and mast-cell tumors in dogs [45]. Mostly however, the molecular alterations involved in cancer development en progression in dogs have not yet been identified. This is also the case for cortisol-secreting ATs. For human ATs, many of the molecular alterations that contribute to pathogenesis have been identified in the past decade. Frequently found alterations include: activation of the cAMP – PKA pathway, for instance due to mutations in PKA regulatory subunit 1 A (*PRKAR1A*) [46], ectopic or increased eutopic hormone receptor expression [47], enhanced steroidogenic factor 1 (*SF-1*) expression [48], increased angiogenesis due to angiopoietin 2 (*ANGPT2*) or vascular endothelial growth factor (*VEGF*) overexpression [49], Wnt-pathway activation, mostly due to β-catenin mutations [50], and activation of IGF – PI3K signaling, for instance due to *IGF-II* overexpression or mutations in tumor suppressor phosphatase and tensin homolog (*PTEN*) [22]. None of these alterations have been addressed in canine cortisol-secreting ATs yet, which impairs the implementation of comparative oncology as a strategy in ACC research.

Conclusions

When compared to the mouse as the classic model for human cancer, using pet dogs as a model for human ACC offers several advantages. Adrenal morphology and function in dogs and humans are highly homologous, and unlike mice, both humans and dogs spontaneously develop cortisol-secreting ATs. While the clinical and

pathological characteristics of cortisol-secreting ACC are comparable, the higher incidence of cortisol-secreting ATs in dogs helps to facilitate research. Recent studies have shown that dogs with cancer can be of great value as a model for human cancer, both to evaluate new therapeutic options and to help illuminate tumor pathogenesis. Further research is needed to determine whether canine and human cortisol-secreting ATs not only share the major clinical characteristics, but also the major mechanisms and mutations thought to be involved in molecular carcinogenesis, such as increased angiogenesis, alterations in the cAMP-PKA signaling pathway, enhanced SF-1 expression, hormone receptor expression and activation of the Wnt and IGF-PI3K signaling pathways. If this is the case, dogs with spontaneous cortisol-secreting ATs might prove to be a valuable model system for human ACC. In addition, this research may, simultaneously help to improve the health and treatment of “man’s best friend”.

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Chapter 3

Activating mutations of GNAS *in canine cortisol-secreting adrenocortical tumors*

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Abstract

Background: Cushing's syndrome or hypercortisolism is a common endocrinopathy in dogs. In approximately 15% of cases the disorder is due to adrenocorticotropin (ACTH)-independent hypersecretion of cortisol by an adrenocortical tumor (AT). Without other explanation, the cortisol hypersecretion has been referred to as autonomous.

Objectives: To investigate whether ACTH-independent hypersecretion of cortisol may be associated with aberrant activation of the melanocortin 2 receptor (MC2R)-cyclic AMP (cAMP)-protein kinase A (PKA) pathway.

Animals: All analyses were performed on 44 cortisol-secreting ATs (14 adenomas and 30 carcinomas) derived from dogs diagnosed with ACTH-independent hypercortisolism.

Methods: Mutation analysis was performed of genes encoding the stimulatory G protein alpha subunit (*GNAS*), *MC2R* and PKA regulatory subunit 1A (*PRKAR1A*) in all ATs.

Results: Approximately one-third of all ATs harbored an activating mutation of *GNAS*. Missense mutations, known to result in constitutive activation, were present in codon 201 in 11 ATs, in codon 203 (1 AT) and in codon 227 (3 ATs). No functional mutations were found in *MC2R* and *PRKAR1A*.

Conclusions and clinical importance: Activation of cAMP signaling is a frequent event in canine cortisol-secreting ATs and may play a crucial role in both ACTH-independent cortisol production and tumor formation. To the best of our knowledge, this is the first report of potentially causative mutations in canine cortisol-secreting ATs.

Introduction

Cushing's syndrome or hypercortisolism is relatively common in dogs, with an estimated incidence of approximately 1 to 2 cases per 1000 dogs per year. In approximately 15% of cases, this disorder is due to a cortisol-secreting adrenocortical tumor (AT) [1,2]. Clinical signs of such a tumor include centripetal obesity, atrophy of muscles and skin, exercise intolerance, polyphagia, polyuria, and polydipsia [3,4], and are a consequence of ACTH-independent hypersecretion of cortisol.

In the healthy adrenal cortex, cell proliferation and steroidogenesis are regulated by melanocortin 2 receptor (MC2R) signaling. Upon ACTH binding to the MC2R, the stimulatory G protein alpha subunit (Gsalpha) activates adenylyl cyclase, producing cAMP. This, in turn, induces protein kinase A (PKA) activity, which results in activation of transcription factors such as cAMP response elements (CREB) that mediate ACTH effects and induce target gene transcription (Fig. 1) [5]. Aberrant activation of the MC2R-cAMP-PKA pathway therefore may be a cause of ACTH-independent hypersecretion of cortisol by ATs.

Despite extensive search, no activating mutations of the *MC2R* have ever been described [6-8]. Mutations that constitutively activate cAMP production mimic MC2R activation in their effects. The best known example is the gsp-oncogene, which arises from a mutation in the stimulatory G protein alpha subunit gene (*GNAS*), and leads to activation of Gsalpha [9,10]. Activating *GNAS* mutations cause McCune-Albright syndrome in humans [11] and also occur in various endocrine tumors, for instance growth hormone-secreting pituitary tumors in humans and thyroid tumors in humans and cats [12-14]. However, only a few cases of activating *GNAS* mutations have been described in adrenocortical adenomas of humans [9,15,16] and no activating *GNAS* mutations have been described in dogs.

Inactivating mutations of the gene encoding PKA regulatory 1 alpha (*PRKAR1A*) subunit cause increased basal and cAMP-stimulated PKA activity [17,18]. Inactivating germ line mutations of this gene are found in approximately two-thirds of people with Carney complex [19], in whom endocrine tumors are common. The most common endocrine gland manifestation in affected people is ACTH-independent hypercortisolism due to primary pigmented nodular adrenocortical disease (PPNAD) [19]. Inactivating *PRKAR1A* mutations also are a relatively common finding in sporadic cortisol-secreting adenomas of humans [20]. In dogs, 1 case report describes a syndrome similar to human Carney complex, but no mutations in PRKAR1A have ever been detected in dogs [21].

Although the knowledge of canine ATs has expanded considerably in recent years, the molecular origin of these adrenocortical neoplasms and the mechanism behind their autonomous cortisol production still are largely unknown, and the role of the MC2R-cAMP-PKA signaling pathway has never been addressed. Therefore, we report here the results of mutation analysis of the full cDNA sequences of *MC2R*, *GNAS* and *PRKAR1A* in 44 canine cortisol-secreting ATs.

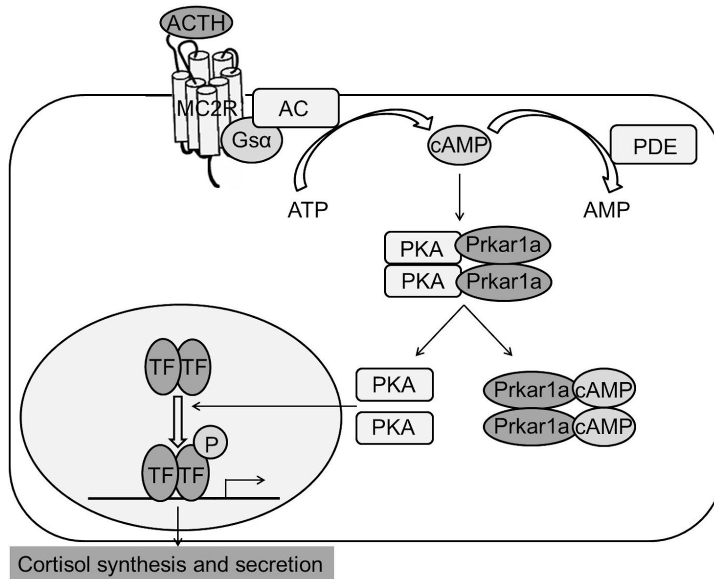

Figure 1

Schematic overview of the MC2R-cAMP-PKA pathway. Binding of ACTH to the MC2R activates adenylate cyclase (AC) through Gsα. AC converts ATP to cAMP, which separates protein kinase A (PKA) from its regulatory subunit PRKAR1A, enabling it to phosphorylate (P) and subsequently activate transcription factors (TF). Phosphodiesterase (PDE) inhibits the pathway by converting the active cAMP to inactive AMP. Abbreviations: MC2R: melanocortin 2 receptor, Gsα: stimulatory G protein alpha subunit, ATP: adenosine triphosphate, cAMP: cyclic adenosine monophosphate, AMP: adenosine monophosphate, PRKAR1A: protein kinase A regulatory subunit 1A

Materials and methods

Animals and Tests

The study included 44 canine cortisol-secreting ATs and 2 normal adrenal glands (whole tissue explants). Normal adrenal glands were obtained from healthy Beagle dogs, euthanized for reasons unrelated to the present study and for which approval was obtained from the Ethical Committee of Utrecht University.

All ATs were derived from patients referred to the Department of Clinical Sciences of Companion Animals of the Faculty of Veterinary Medicine in Utrecht between 2001 and 2012. Suspicion of hypercortisolism was based on the history, physical examination findings and routine laboratory findings. The diagnosis of ACTH-independent hypercortisolism due to an AT was based upon (i) increased urinary cortisol secretion, which was not suppressible with a high dose of dexamethasone, (ii) suppressed or undetectable basal plasma ACTH concentrations [2] and (iii) demonstration of an AT by ultrasonography, computed tomography or both [22]. All ATs were removed by unilateral adrenalectomy. The dogs' ages at the time of surgery ranged from 2 to 13 years (mean, 9 years). Six dogs were mongrels and the others were of 26 different breeds. Twenty-two of the dogs were male (10 castrated) and 22 female (15 spayed). Permission to use AT tissue for this study was obtained from all patient owners, and the study was approved by the Ethical Committee of Utrecht University.

Histopathology

Histopathological evaluation of ATs was performed on formalin-fixed and paraffin-embedded tissue slides of all samples and used to confirm the diagnosis and to classify the tumors. All histological evaluations were performed by a single pathologist. Classification was based on the criteria described by Labelle *et al* [23]. Classification as a carcinoma was based on histological evidence of vascular invasion, peripheral fibrosis, capsular invasion, trabecular growth, hemorrhage, necrosis, and single cell necrosis. Typical histological characteristics of adenomas were hematopoiesis, fibrin thrombi, and cytoplasmic vacuolization. Based on these criteria, the tumor group consisted of 14 adenomas and 30 carcinomas.

Total RNA extraction and reverse transcription

Tissue fragments for RNA isolation were snap frozen in liquid nitrogen within 10-20 minutes after surgical removal. Total RNA was isolated from the samples using the RNeasy mini kit (Qiagen, Hilden, Germany), according to manufacturer's protocols. A DNAse step was performed to avoid DNA contamination. RNA concentrations were measured on the NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE). cDNA synthesis was performed using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA), according to manufacturer's protocols. For all samples, one cDNA reaction was performed without Reverse Transcriptase (RT-), to check for contamination with genomic DNA.

PCR

Primers for PCR were designed using Perl-primer v1.1.14 according to the parameters in the Bio-Rad iCycler manual, and ordered from Eurogentec (Maastricht, the Netherlands). Forward primers were located in the 5' untranslated region (UTR) of the genes of interest, whereas reverse primers were located in the 3'UTR. For the *MC2R*, the canine UTR sequences were not available, and were predicted based on the human UTR sequences and the canine genomic sequence. Overlapping primer pairs were used when a gene could not be amplified in 1 stretch. For all primer pairs, a PCR temperature gradient was performed to determine the optimal annealing temperature.

PCR primers	Sequence (5'-3')	Location	T_m	Product length
MC2R Fw 69	CGAGGCAGAGTAACACCT	-41/-24	55°C	674
MC2R Rv 743	GGAAGCGTCAAGATCTTCC	614/632		
MC2R Fw 410	CACAGCGGATGACATTATGG	300/319	55°C	781
MC2R Fw 1190	AAGCATGAGCATTTGTTGGT	1061/1080		
GNAS Fw 352	CCATGGGCTGCCTCGGAAACA	352/372	56°C	1357
GNAS Rv 1708	TTAAGCAAGCGGAAGGGAAGAAA	1686/1708		
PRKAR1A Fw 20	GCTATCGCGGAGTAGAG	20/36	59°C	1336
PRKAR1A Rv 1355	AGAGGAAGAGAAAGCAGTC	1337/1355		

Table 1

PCR primers for the amplification of canine MC2R, GNAS and PRKAR1A. All positions are based on the mRNA sequence, as published on the NCBI website. Accession numbers used: MC2R: XM_003638756.1, GNAS: NM_001003263.1, PRKAR1A: XM_537577.3.

Abbreviations: MC2R: melanocortin 2 receptor, GNAS: stimulatory G protein alpha subunit, PRKAR1A: protein kinase A regulatory subunit 1A, Fw: Forward primer, Rv: Reverse primer

Formation of the proper PCR products was evaluated by gel electrophoresis, to check for the correct product length. In case of correct product lengths, a sequencing reaction was performed to confirm the identity of the transcript, using the ABI3130XL Genetic analyzer (AB applied biosystems, Carlsbad, CA) according to the manufacturer's protocol. After optimization of the protocol, the complete cDNA of all target genes was amplified in all ATs. PCR reactions were performed using Phusion® Hot Start Flex DNA Polymerase (New England BioLabs Inc., Ipswich, MA) on a Dyad Disciple™ Peltier Thermal Cycler (BioRad) for *PRKAR1A* and on a C1000 Touch thermal cycler (BioRad) for *MC2R* and *GNAS*. All PCR primers and their characteristics are listed in Table 1.

Sequencing

All sequence primers were designed using Perl-primer v1.1.14, and ordered from Eurogentec. Primers were located every 300-500 base pairs along the entire transcript, or closer together when additional primers were needed for complete coverage. All PCR primers also were used as sequence primers. PCR products were amplified for sequencing using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and filtrated using Sephadex G-50 Superfine (Amersham, Buckinghamshire, United Kingdom). Sequencing reactions were performed on an ABI3130XL Genetic analyzer, according to manufacturer's instructions. The obtained sequences were compared to the consensus mRNA sequence using DNAstar Lasergene core suite 9.1 SeqMan software. All mutations affecting the amino acid sequence were confirmed by repeat RNA extraction, and sequenced in both sense and antisense directions. All sequence primers and their characteristics are listed in Table 2.

Sequence primers	Sequence (5'-3')	Location
MC2R Fw 810	CCCTTTGTCCTTCATGTTCTG	700/720
MC2R Rv 340	TATACAAGCTGCCCATCATATCAG	206/229
GNAS Fw 777	TCCCTCCTGAGTTCTATGAG	778/797
GNAS Fw 1226	AACAAGCAAGACCTGCTC	1127/1244
GNAS Rv 845	CTCATAGCAGGCACGCACTCC	825/845
GNAS Rv 951	CAGCGAAGCAGATCCTG	936/952
GNAS Rv 1504	CTGAATGATGTCACGGCA	1488/1505
PRKAR1A Fw 174	GGGAATGTGAGCTCTATGTC	175/194
PRKAR1A Fw 329	CTGCAGAAAGCAAGCTCC	330/347
PRKAR1A Fw 575	TTTGATGCCATGTTTCCAG	576/594
PRKAR1A Fw 750	GAACACCTAGAGCAGCCA	751/768
PRKAR1A Fw 975	TGGTACAGGGAGAACCAG	976/993
PRKAR1A Rv 452	CTCCTCCGTGTAGACTTCG	434/452
PRKAR1A Rv 699	CCCATTCGTTGTTGAC	684/699
PRKAR1A Rv 855	GCTTTCTCAGAGTGCTTCC	837/855
PRKAR1A Rv 975	CTGGTTCTCCCTGTACCA	975/993
PRKAR1A Rv1255	AAACTGTTGTACTGCTGGA	1237/1255

Table 2

Sequencing primers for the mutation analysis of canine MC2R, GNAS and PRKAR1A. All positions are based on the mRNA sequence, as published on the NCBI website. Accession numbers used: MC2R: XM_003638756.1, GNAS: NM_001003263.1, PRKAR1A: XM_537577.3.

Abbreviations: MC2R: melanocortin 2 receptor, GNAS: stimulatory G protein alpha subunit, PRKAR1A: protein kinase A regulatory subunit 1A, Fw: Forward primer, Rv: Reverse primer

Results

Mutation analysis of *MC2R* identified 3 different silent point mutations and 1 amino-acid changing (missense) point mutation. The silent mutations or single nucleotide polymorphisms (SNPs) found in codon 38 (GGG>GGA), codon 237 (GCG>GCC) and codon 286 (GCG>GCA) were present in 8, 21 and 21 ATs, respectively, and occurred both in hetero- and homozygous form. The missense mutation, a V291I substitution, was present in 3 of the 44 ATs (2 carcinomas, 1 adenoma) and was present only in heterozygous form (Fig. 2A).

Mutation analysis of GNAS showed the presence of a splice variant, 1 silent point mutation and 7 different missense mutations. The splice variant of GNAS, in which exon 3 is missing, is analogous to the human GNAS transcript variant 3 (GenBank: NM_080426.2) or GNAS-short (GNASS). It was present in all ATs and normal adrenal glands, alongside the full length transcript. The silent mutation was found in codon 201 (CGT>CGC) and was present in 8 ATs in both hetero- and homozygous form. Missense mutations were present in 14 of the 44 ATs, including 4 of the 14 adenomas and 10 of the 30 carcinomas. All missense mutations were heterozygous. Eleven of the 14 missense mutations were located in codon 201 (Fig. 2B). They were present in 8 carcinomas and 3 adenomas and comprised the following substitutions: R201C (5×), R201H (4×), R201S (1×) and R201L (1×). A missense mutation in codon 203 (L203P) was present in 1 adenoma (Fig. 2C). Missense mutations in codon 227 (Fig. 2D) were present in 2 carcinomas (Q227H and Q227R). An overview of the different missense mutations is presented in Table 3.

Mutation analysis of PRKAR1A showed the presence of 2 different silent mutations. A silent mutation in codon 317 (AGA>CGA) was present in 4 carcinomas and a silent mutation in codon 311 (GAG>GAA) was present in 1 adenoma. Mutations that changed the amino acid sequence were not found in any of the ATs.

Discussion

In this study, *GNAS* mutations were detected in 14 of the 44 cortisol-secreting ATs of dogs, whereas no functional mutations were found in *MC2R* and *PRKAR1A*. All *GNAS* mutations detected in the ATs of these dogs previously have been described in the human literature, and have been found to cause constitutive activation of cAMP signaling [14,24-26]. Although additional in vitro assays would be necessary to establish a causal relationship, our results strongly suggest the involvement of

increased cAMP signaling, caused by activating *GNAS* mutations, in the pathogenesis of a subset of cortisol-secreting ATs in dogs. This finding even may provide an explanation for autonomous, ACTH-independent, cortisol secretion in the affected subset of ATs.

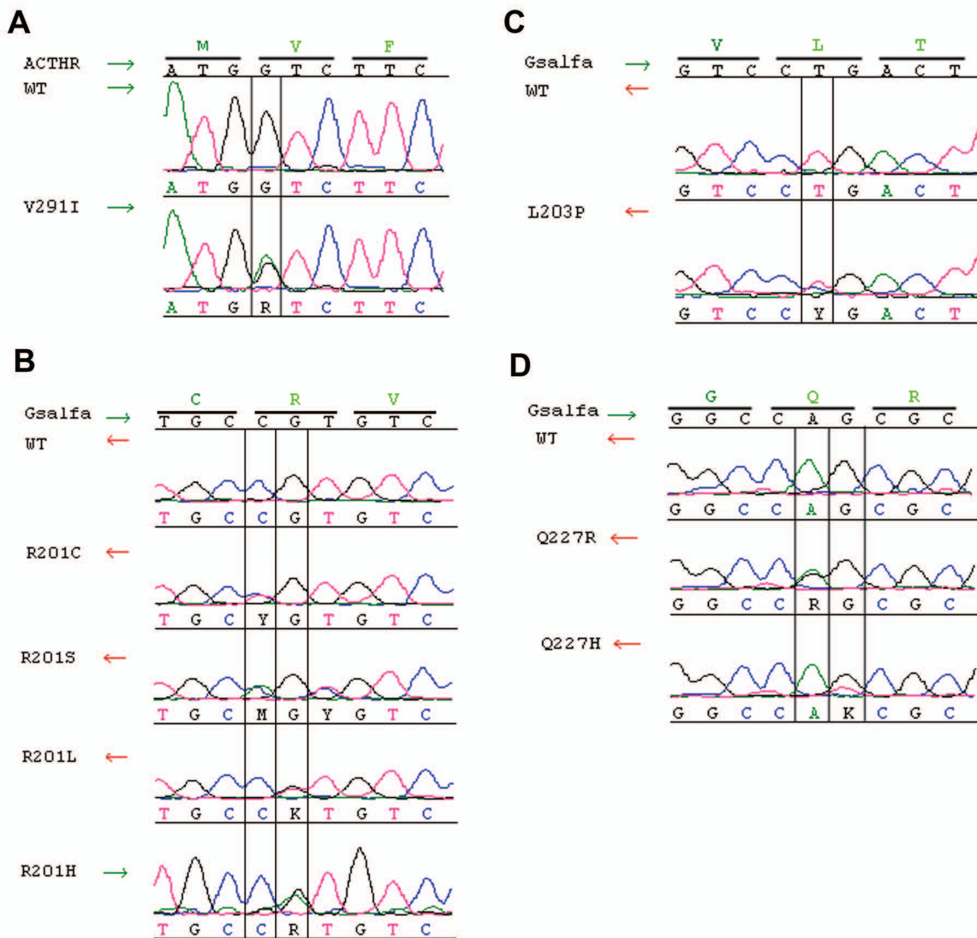

Figure 2

Representative examples of the mutations found in codon 291 of the MC2R (A) and in codons 201 (B), 203 (C) and 227 (D) of GNAS in canine cortisol secreting ATs. Reference sequences are based on XM_003638756.1 (MC2R) and NM_001003263.1 (GNAS). WT: wild type.

Abbreviations: MC2R: melanocortin 2 receptor, GNAS: stimulatory G protein alpha subunit

Mutation	Nucleotide	Codon	Basepair change	Amino acid change	Number of ATs
Arg201Cys	954	201	**C**GT>**T**GT	Arg>Cys	5
Arg201His	955	201	C**G**T>C**A**T	Arg>His	4
Arg201Ser	954	201	**C**GT>**A**GT	Arg>Ser	1
Arg201Leu	955	201	C**G**T>C**T**T	Arg>Leu	1
Gln227His	1034	227	CA**G**>CA**T**	Gln>His	1
Gln227Arg	1033	227	C**A**G>C**G**G	Gln>Arg	1
Leu203Pro	961	203	C**T**G>C**C**G	Leu>Pro	1

Table 3

Overview of all missense mutations of GNAS in 44 canine cortisol secreting ATs. All nucleotide positions are based on the mRNA sequence (NM_001003263.1), as published on the NCBI website. All amino acid positions are based on the protein sequence (NP_001003263.1), as published on the NCBI website. Abbreviations: GNAS: stimulatory G protein alpha subunit

In cortisol-secreting ATs of humans, activation of the cAMP signaling pathway is a well-known phenomenon, however, activating *GNAS* mutations in these tumors are extremely rare, and only have been described in benign lesions [9,15,16]. Activating *GNAS* mutations in humans are associated with McCune Albright syndrome (MAS), in which they result in macronodular hyperplasia of the adrenal glands and hypercortisolism [11]. *GNAS* mutations also have been detected in pituitary and pancreatic tumors of humans and in thyroid tumors of humans and cats [12-14]. In humans, substitutions of Arg^{201} are most common, followed by Gln^{227} substitutions [11,13,14] whereas Arg^{201} and Gln^{227} also were the affected codons in thyroid tumors of cats [12]. Likewise, in our canine cohort most of the mutations were substitutions of Arg^{201} and Gln.^{227}

Four possible substitutions of Arg^{201} in *GNAS* have been described in humans, in decreasing occurrence: R201C, R201H, R201S and R201L [11,24,26]. Of these mutations only R201C and R201H previously have been reported in the adrenal cortex, including cortisol-secreting ATs [15,27,28]. In cats, R201C is the only known mutation affecting Arg^{201} [12]. In our canine AT cohort, all 4 known Arg^{201} mutations were identified, with a higher frequency of R201C and R201H mutations. Five different substitutions have been described at the second hotspot for human *GNAS* mutations (i.e.,Gln^{227}) of which Q227H, Q227L and Q227R are most common [14,24]. A single report of a Q227H substitution in a cortisol-secreting adrenocortical adenoma in a human has been published [9]. In thyroid tumors of cats, both Q227R and Q227L have been reported [12]. In our canine AT cohort, both Q227H and Q227R substitutions were detected. The L203P substitution at Leu^{203} found in 1 AT in a dog has only been described in a thyroid tumor of a human [25].

The *GNAS* splice variant that was present in all samples was analogous to human transcript variant 3 [29]. This transcript variant corresponds to a shorter Gsalpha protein (GNASS), which was found to be co-expressed with the long variant (GNASL) in nearly all cell types, although the relative amounts vary depending on the tissue type. In the adrenal cortex of humans, GNASL was found to be the predominant isoform [30]. Both variants induce cAMP production, and some investigations have indicated differences in their activity, affinity of guanidine diphosphate (GDP) binding and receptor interaction [31-33]. However, whether these differences result in clinically relevant biological effects still is unclear [34]. Moreover, the presence of both variants in all ATs and normal adrenal glands in our canine cohort, makes a causal role in canine adrenocortical tumorigenesis unlikely.

cAMP is the main cellular signal for inducing cortisol secretion [5]. Therefore, the activating *GNAS* mutations in ATs of dogs, resulting in constitutive cAMP production, may explain the ACTH-independent cortisol production for this subgroup. Apart from cortisol production, increased cAMP signaling also is known to play a role in adrenal tumorigenesis [35]. Activating mutations in *GNAS* induce tumor formation in cAMP-sensitive tissue types by increasing cell proliferation. The mutated *GNAS* thus is referred to as the gsp oncogene [36]. *GNAS* activating mutations have been shown to result in induction of mitogen-activated protein kinase (MAPK or ERK) and p53 signaling, focal adhesion kinase (FAK) pathways and nuclear factor kappa-B (NFKB) expression [28]. Both P53 and Ras-Raf-MAPK pathways are well known for their roles in carcinogenesis and have been implicated as factors in adrenal tumorigenesis [37,38]. The FAK pathway and NFKB also have been implicated as factors in the pathogenesis of various tumor types [39,40]. Therefore, it is likely that the activating *GNAS* mutations found in ATs of dogs play a role in tumorigenesis.

In contrast to the high prevalence of activating *GNAS* mutations in cortisol-secreting ATs of dogs, no mutations affecting the amino acid sequence were found in *PRKAR1A*. This contrasts with the important role of *PRKAR1A* mutations in adrenal pathology in humans and mice. Mutations in *PRKAR1A* cause Carney complex in humans, with PPNAD as 1 of the main consequences [19] When cAMP-PKA activation is present in ATs of humans, it nearly always originates from *PRKAR1A* mutations or other PKA signaling abnormalities [35]. Likewise, in the mouse, *PRKAR1A* inactivation and AT formation are closely linked. Transgenic mice lacking PRKAR1A activity in the adrenal cortex develop ACTH-independent Cushing’s syndrome [41] and activated PKA signaling due to PRKAR1A loss of function results in a tumor formation syndrome similar to human Carney complex [42,43].

In dogs, a single case report describes a syndrome similar to human Carney complex, but in this dog *PRKAR1A* was not altered [21]. The absence of missense *PRKAR1A* mutations in our canine AT cohort, combined with the fact that no mutations in PRKAR1A have ever been detected in dogs, appear to indicate a difference in the molecular origin of cAMP-PKA activation between adrenal glands of dogs and their human and murine counterparts. The pathways affected by cAMP-PKA activation have been shown to differ depending on the molecular origin of the activation. cAMP-PKA activation due to *PRKAR1A* mutations stimulates a different set of cellular pathways and target genes than activation caused by *GNAS* mutations [28]. Both *PRKAR1A* and *GNAS* mutations activate MAPK and P53 signaling, whereas the FAK pathway and NFKB specifically are induced by *GNAS* mutations, and *PRKAR1A* mutations induce activation of the Wnt-pathway, 1 of the main cellular pathways implicated in AT pathogenesis in humans [28,44]. However, although differences exist between germ line *PRKAR1A* defects and somatic *GNAS* mutations, dogs and humans still share common MAPK and p53 signaling pathway activations that might be important targets for therapy in both species.

Mutation analysis of the *MC2R* identified the presence of a V291R missense mutation in 3 ATs, which has not been described previously in the literature. However, this substitution is not likely to have a functional effect on the receptor, because valine and isoleucine are alike in polarity and charge. Moreover, in the human MC2R, isoleucine and not valine is the consensus amino acid. Otherwise, no mutations in the *MC2R* were found, corresponding to the situation in humans, where activating *MC2R* mutations have never been identified.

In conclusion, this study demonstrates the presence of activating *GNAS* mutations in a large portion of both benign and malignant cortisol-secreting ATs in dogs. These results strongly suggest increased cAMP signaling as a factor in the pathogenesis of these tumors and may explain the autonomous secretion of cortisol in the affected subset of ATs. This study thus is the first to identify potentially causal mutations in cortisol-secreting ATs of dogs.

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Chapter 4

Expression of somatostatin, dopamine, progesterone and growth hormone receptors in canine cortisol-secreting adrenocortical tumors

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Abstract

Cortisol-secreting adrenocortical tumours (AT) in dogs are characterized by uncontrolled growth and excessive cortisol secretion. Alterations in hormone receptor expression may be involved in tumor growth and hypersecretion of cortisol. Therefore, the relative mRNA expression of growth hormone receptor, progesterone receptor, somatostatin receptors 1-3 and dopamine receptors (DRD1-2 and DRD5) was evaluated in 36 ATs and 15 normal adrenal glands. Compared to normal adrenals, DRD2 expression was lower in carcinomas, while SSTR1 expression was lower in both adenomas and carcinomas. Both might have contributed to loss of inhibition of tumor growth and cortisol secretion. In ATs with recurrence within 2.5 years, a markedly increased expression of DRD1 was detected. The detection of hormone receptor mRNA in all canine ATs may appear useful in targeted therapeutic intervention.

Cortisol-secreting adrenocortical tumors (ATs) are relatively common in dogs and are characterized by uncontrolled growth and excessive cortisol secretion, the molecular mechanisms of which are unknown in the majority of cases [1]. Altered expression of hormone receptors could provide an explanation for uncontrolled tumor growth and excessive cortisol secretion, and also represents an opportunity for targeted treatment. Increased expression of growth hormone receptor (GHR) and progesterone receptor (PR) has been detected in human cortisol-secreting ATs [2], which may indicate an autocrine PR-GHR mechanism like in canine mammary carcinomas. Overexpression of somatostatin receptors (SSTR1-5) [3], and presence of D2-like dopamine receptors (DRD2 and DRD4) have also been reported in human ATs [4]. These receptors exert anti-proliferative and anti-secretory effects through G-protein mediated inhibition of cAMP signaling [5,6]. The aim of this study was to evaluate the mRNA expression of SSTRs, DRs, GHR and PR in canine cortisol-secreting ATs.

Patient material was derived from 36 dogs with cortisol-secreting ATs, that underwent adrenalectomy at the Department of Clinical Sciences of Companion Animals. The diagnosis of ACTH-independent hypercortisolism due to a cortisol-secreting AT was made as described previously [7]. Permission to use the AT tissue was obtained from all patient owners. The dogs' age at the time of surgery ranged from 2 to 13 years (mean, 9 years). Seven dogs were mongrels and the other dogs were of 22 different breeds. Eighteen of the dogs were male (eight castrated) and 18 female (12 neutered). Fifteen archived whole tissue explants of normal canine adrenal glands were available as controls.

Histopathological evaluation by a single pathologist was used to confirm the diagnosis and classify the tumors [7]. The tumor group consisted of 11 adenomas and 25 carcinomas. Follow-up information was available for 15 dogs. In seven dogs recurrence of hypercortisolism was confirmed by endocrine testing within 2.5 years after surgery. The remaining eight dogs were in remission for at least 2.5 years after adrenalectomy.

Total RNA isolation and cDNA synthesis were performed as previously described [7]. Primers were designed to detect the mRNA expression levels of GHR, PR, SSTR1-3 and 5 and DRD1, D2 and D5 (Table 1). Primer design, qPCR optimization and mRNA expression analysis were performed according to previously described protocols [7]. Ribosomal protein S5 (RPS5), ribosomal protein S19 (RPS19), signal recognition particle receptor (SRPR) and beta-glucuronidase (GUSB) were used as reference genes [8]. The relative expression of the reference genes revealed no significant differences between groups and reference gene expression was shown to be stable

using GeNorm software. Target gene expression was deemed to be below the detection threshold if no specific PCR product could be detected after 40 cycles.

Statistical analyses were performed using SPSS20 (IBM). The Mann-Whitney U test was used to compare relative mRNA expression levels between adrenocortical adenomas versus normal adrenals and adrenocortical carcinomas versus normal adrenals. Additionally, mRNA expression in ATs with and without recurrent disease within 2.5 years were compared. For the first comparison, Bonferroni correction was applied and $P < 0.025$ was considered significant, while for the latter comparison, $P < 0.05$ was considered significant.

qPCR primers		Sequence (5'-3')	T_m	Product length (bp)
cf_GHR	*Fw*	GCG CAT CCC AGA GTC TAC A	58 °C	133
	Rv	ACC ATG ACG AAC CCC ATC T		
cf_PR	*Fw*	CAA TGG AAG GGC AGC ATA AC	57 °C	102
	Rv	CAG CAC TTT CTA AGG CGA CA		
cf_SSTR1	*Fw*	GTT CAC CAG CAT CTA CTG TCT G	66.3 °C	148
	Rv	CAG AAT GAC CAG CAG CGA G		
cf_SSTR2	*Fw*	ACA GAG CCA TAC TAT GAC CTG ACC	60.5 °C	128
	Rv	GTC TTC ATC TTG GCA TAG CGG AG		
cf_SSTR3	*Fw*	AGA ACG CCC TGT CCT ACT G	60.7 °C	89
	Rv	GAA GAT GCT GGT GAA CTG GT		
cf_SSTR5	*Fw*	CGG CAC GCC AAG ATG AAG ACG	65 °C	125
	Rv	CCG AAG GGC CAG TAG GAG ACG		
cf_DRD$_1$	*Fw*	CTC TGA ACA CCT CTA CTA TGG	62.5 °C	145
	Rv	AAC CTG ATG ACA GCG G		
cf_DRD$_2$	*Fw*	TAG GTG AGT GGA AAT TCA GC	58.6 °C	250
	Rv	TTC TGG TCT GTG TTG TTG AG		
cf_DRD$_5$	*Fw*	CGT CTT CGT CTG GTT CG	58 °C	149
	Rv	CAT TGC TGA TGT TCA CTG TC		

Table 1

Quantitative RT-PCR primer pairs for the detection of GH-receptor (GHR), progesterone receptor (PR), somatostatin receptors 1-3 and 5 (SSTR1-3 and 5) and dopamine receptors 1, 2 and 5 (DRD_1, D_2 and D_5). NCBI accession numbers used: GHR: NM_001003123.1, PR: NM_001003074.1, SSTR1: NM_001031816.1, SSTR2: NM_001031817.1, SSTR5: NM_001286852.1, DRD_1: XM_005619193.1, DRD_2: NM_001003110.1, DRD_5: XM_005618578.1

Abbreviations: Fw: Forward primer, Rv: Reverse primer

For DRD5, SSTR3 and SSTR5, mRNA expression was below the detection threshold in a large proportion of samples, therefore these genes were excluded from further analysis. In all ATs and normal adrenals, mRNA encoding GHR, PR, SSTR1 and 2 and DRD1 and D2, mRNA was detected. Carcinomas exhibited a significantly lower relative mRNA expression of DRD2 (0.5-fold, P =0.002) and SSTR1 (0.4-fold, P <0.001) when compared to normal adrenal glands. In adenomas, only SSTR1 expression was significantly lower (0.3-fold, P <0.001) (Fig. 1A). No significant differences were detected in the relative expression of GHR, PR, SSTR2 and DRD1.

In dogs with recurrent disease within 2.5 years, a significantly higher expression of DRD1 (4.2-fold, P =0.013) was detected (Fig. 1B). For none of the other target genes significant differences in relative mRNA expression were detected.

The results of this study show that GHR, PR, SSTR1 and SSTR2 and DRD1 and DRD2, mRNA could be detected in all normal adrenals and ATs. However, in contrast to what is known in humans, no hormone receptor overexpression was detected. In carcinomas, the relative mRNA expression of DRD2 and SSTR1 was even significantly lower, and the same held true for SSTR1 in adenomas. Decreased DRD2 or SSTR1 expression may reflect an escape from the anti-secretory and anti-proliferative effects of somatostatin and dopamine signaling. The lack of changes in relative expression for GHR, PR, DRD1 and SSTR2 in this study does not support a role for these receptors in the development of ACTH-independent hypercortisolism or uncontrolled AT growth.

Interestingly, DRD1 expression was significantly higher in carcinomas with recurrence within 2.5 years. DRD1 is mainly associated with G-protein stimulatory subunit alpha (Gsα) induced cAMP production [5]. Aberrant cAMP signaling plays an important role in the development of human ACTH-independent hypercortisolism and adrenal tumorigenesis [9] and in dogs we have detected activating mutations of Gsα in approximately one third of cortisol-secreting ATs [10].

In conclusion, the enhanced DRD1 expression in carcinomas with recurrent disease within 2.5 years may represent an alternative mechanism for cAMP signaling activation, which is known to play a role in AT pathogenesis. Additionally the detection of mRNA encoding GHR, PR, SSTR1 and SSTR2 and DRD1 and DRD2 in all samples, indicates the presence of these receptors in canine cortisol-secreting ATs, which may be the basis to explore the possibility of targeted therapeutic intervention.

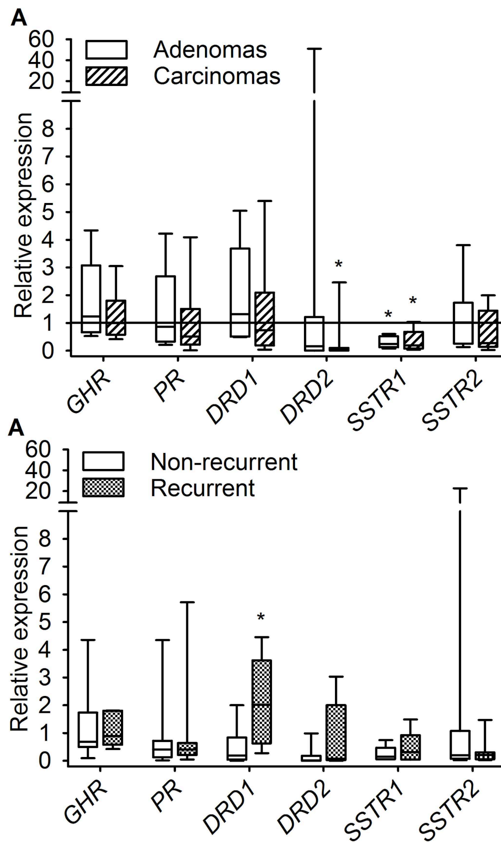

Figure 1

Box-and-whisker plot depicting the relative mRNA expression of GH receptor (GHR), progesterone receptor (PR), somatostatin receptors (SSTR) 1, 2 and 5 and dopamine receptors (DRD_1, DRD_2 and DRD_5) in 36 canine ATs (11 adenomas and 25 carcinomas) and 15 normal adrenal glands. (A) Relative expression in adenomas and carcinomas when compared to normal adrenal glands. (B) Relative expression in ATs with recurrence within 2.5 yr after adrenalectomy when compared with ATs remaining in remission for at least 2.5 yr. Significant changes ($P < 0.025$ for A and $P < 0.05$ for B) are marked with an asterisk.

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Chapter 5

Expression of Steroidogenic Factor 1 in canine cortisol-secreting adrenocortical tumors and normal adrenals

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Abstract

We report on a screening for the relative mRNA and protein expression of steroidogenic factor 1 (SF-1) in normal canine adrenals (n=10) and cortisol-secreting adrenocortical tumors (11 adenomas and 26 carcinomas). The relative mRNA expression of SF-1 was determined by quantitative RT-PCR analysis and revealed no differences between normal adrenals, adenomas, and carcinomas. Immunohistochemistry demonstrated SF-1 protein expression in a nuclear pattern throughout the normal adrenal cortex and a predominantly nuclear staining pattern in adrenocortical tumors. Of the 15 dogs available for follow up, 7 dogs developed hypercortisolism within 2.5 yr after adrenalectomy, with metastatic disease in 6 dogs and adrenocortical tumor regrowth in 1 dog. The relative SF-1 mRNA expression in dogs with early recurrence was greater (2.46-fold, P = 0.020) than in dogs in remission for at least 2.5 yr after adrenalectomy. In conclusion, we demonstrated the presence of SF-1 expression in normal canine adrenals and adrenocortical tumors. The high SF-1 mRNA expression in carcinomas with early recurrence might indicate its value as a prognostic marker, as well as its potential for therapeutic development.

Introduction

Canine cortisol-secreting adrenocortical tumors (ATs) underlie ACTH-independent hypercortisolism and are characterized by uncontrolled growth and hormone secretion [1]. Their pathogenesis is largely unknown. Potential explanations for ACTH-independent hypersecretion of cortisol and growth of canine ATs can be derived from the current understanding of adrenal growth biology.

Adrenal development and steroidogenesis depend greatly on the expression of steroidogenic factor 1 (SF-1) [2]. Mice with homozygous null mutations in SF-1 are born without adrenal glands and gonads and die within hours after birth due to adrenal insufficiency [3,4]. In the adult adrenal cortex, SF-1 plays a prominent role in the regulation of steroidogenesis, by being an obligate activator of the majority of cytochrome P450 steroid hydroxylases and steroidogenic acute regulatory (StAR) protein [5,6]. The growth-promoting effect of SF-1 in the adult adrenal gland is dosage-dependent. For the compensatory growth of the contralateral adrenal gland following unilateral adrenalectomy, physiologic SF-1 expression is sufficient [7], while an increased dosage stimulates proliferation and decreases apoptosis in human adrenocortical cells, and triggers adrenal tumorigenesis in mice [8]. In childhood ATs, SF-1 gene amplification and protein overexpression are the most consistent findings [9]. In adult humans with an adrenocortical carcinoma, SF-1 staining intensity is negatively correlated with survival, and SF-1 is considered a tumor stage-independent prognostic factor [10]. Taken together, this indicates that SF-1 plays an important role in the pathogenesis of ATs.

In dogs, the expression of SF-1 has been studied in sex-reversal syndrome only [11]. The aim of the present study was to determine the expression of SF-1 mRNA and protein in normal adrenals and cortisol-secreting ATs of dogs.

Materials and Methods

Animals and tissues

The study was approved by the Ethical Committee of Utrecht University. Cortisol-secreting ATs were obtained from 37 dogs that underwent adrenalectomy at the Department of Clinical Sciences of Companion Animals and permission to use the AT tissue was obtained from all patient owners. The dogs' ages at the time of surgery ranged from 6 to 14 yr (mean, 9 yr). Twelve dogs were mongrels and the others were of 10 different breeds. Eighteen of the dogs were male (8 castrated) and 19 female (15 neutered). The diagnosis of ACTH-independent hypercortisolism was made as

described previously [1, 12]. Ten normal adrenal glands (whole tissue explants) of healthy beagle dogs served as control tissue. The dogs were euthanized for reasons unrelated to the present study. Their ages ranged from 2 to 5 yr, there were 5 males and 5 females, all intact.

From 15/37 dogs, follow up information was available. When reoccurrence of ACTH-independent hypercortisolism was suspected, the diagnosis was confirmed by endocrine testing, measurements of the basal plasma ACTH concentration and diagnostic imaging [1, 12]. The dogs were categorized in (i) a group with relapse of ACTH-independent hypercortisolism within 2.5 yr after surgery and (ii) a group in remission for at least 2.5 yr after surgery.

Histopathology

All tissues were fixed in 4% buffered formalin, embedded in paraffin after at least 24 h and maximally 48 h of fixation, cut into 5 μm sections and mounted on SuperFrost Plus microscope slides (Menzel-Gläser, Braunsweig, Germany). On histopathology, there were 26 carcinomas and 11 adenomas. The diagnosis was made by a single pathologist. In case of doubt, a second pathologist was consulted and slides were reviewed at a multihead microscope, and a consensus was reached. In all carcinomas there was evidence of invasion of neoplastic cells into blood vessels and/or capsular invasion. Additional characteristics for carcinomas were trabecular growth pattern and peripheral fibrosis. In agreement with the criteria published before [13], none of the adenomas exceeded 2 cm diameter at the major axis width, while carcinomas were generally larger. Typical histological characteristics for adenomas were hematopoiesis, fibrin thrombi, and cytoplasmic vacuolization, while hemorrhage, necrosis, and single cell necrosis were detected in both, adenomas and carcinomas.

Total RNA extraction and reverse transcription

Tissue fragments for RNA isolation were snap frozen in liquid nitrogen within 10-20 min after surgical removal and stored at -70°C until RNA isolation. Total RNA isolation and cDNA synthesis were performed, as described previously [12].

Quantitative RT-PCR

Quantitative RT-PCR primers for SF-1 (XM_846937, Fw: AGGGCTGCAAGGGGT TTTTCAA, Rv: CATCCCCACTGTCAGGCACTTCT, T_a: 59 °C) were designed using Perl-primer v1.1.14 according to the Bio-Rad iCycler parameters, and ordered from Eurogentec (Maastricht, The Netherlands). PCR reaction optimization and confirmation of primer specificity were performed as described previously [12].

The mRNA expression abundances of SF-1 were measured in 10 normal adrenals and 37 cortisol-secreting ATs (26 carcinomas and 11 adenomas). All reactions were performed on a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA), according to a previously described protocol [12]. Ribosomal protein S5 (RPS5), ribosomal protein S19 (RPS19) and hypoxanthine-guanine phosphoribosyltransferase (HPRT) were used as reference genes [14]. Analysis of the relative expression abundances of the reference genes revealed no significant differences between groups and their expression was shown to be stable using GeNorm software, justifying their use as reference genes.

Immunohistochemistry

For IHC staining of SF-1, tissue slides were rehydrated in a series of xylene-alcohol baths. Antigen retrieval was performed using 10 mM sodium citrate buffer pH 6, for 20 min at 95°C. To block endogenous peroxidase activity, slides were incubated with peroxidase block (S2003, Dako, Glostrup Denmark) for 5 min. Aspecific binding sites were blocked with 10% normal goat serum in PBS for 20 min. Slides were incubated overnight at 4°C with a polyclonal rabbit-anti-human anti SF-1 antibody (LS- A5534, MBL International, USA), in a 1:200 dilution in 1% normal goat serum in PBS. Subsequently, all slides were incubated with anti-rabbit HRP conjugated secondary antibody (Dako K4003) for 45 min at RT. Antibody detection was performed using Dako K3468 HRP substrate. All slides were incubated with 3,3'-diaminobenzidine (Dako liquid DAB + substrate chromogen system, K3468, Dako, Glostrup, Denmark) for 4 min and subsequently counterstained with heamatoxylin, dehydrated, and mounted. To confirm the specificity of the reaction, blocking peptides against SF-1 were used (LS-P5534, MBL in a concentration of 1 mg/ml and a dilution of 1:400). Pre-incubation of the antibody with those blocking peptides abolished all staining. IHC analysis was performed using light microscopy. The presence and localization (membranous, cytoplasmic or nuclear) of staining in ATs and normal adrenals were described.

Statistical analyses

Statistical analyses were performed with SPSS20 (IBM, Armonk, NY, USA). Relative mRNA expression abundances were calculated using the ΔΔ-Ct method [15]. A Mann-Whitney U test was used to compare the relative expression abundances of SF-1 between normal adrenals, adenomas and carcinomas, and between dogs with recurrence of hypercortisolism within 2.5 yr and in dogs in remission for at least 2.5 yr after adrenalectomy. For the first comparison, a Bonferroni correction was applied and $P < 0.025$ was considered significant, while for the latter comparison, a P value < 0.05 was considered significant.

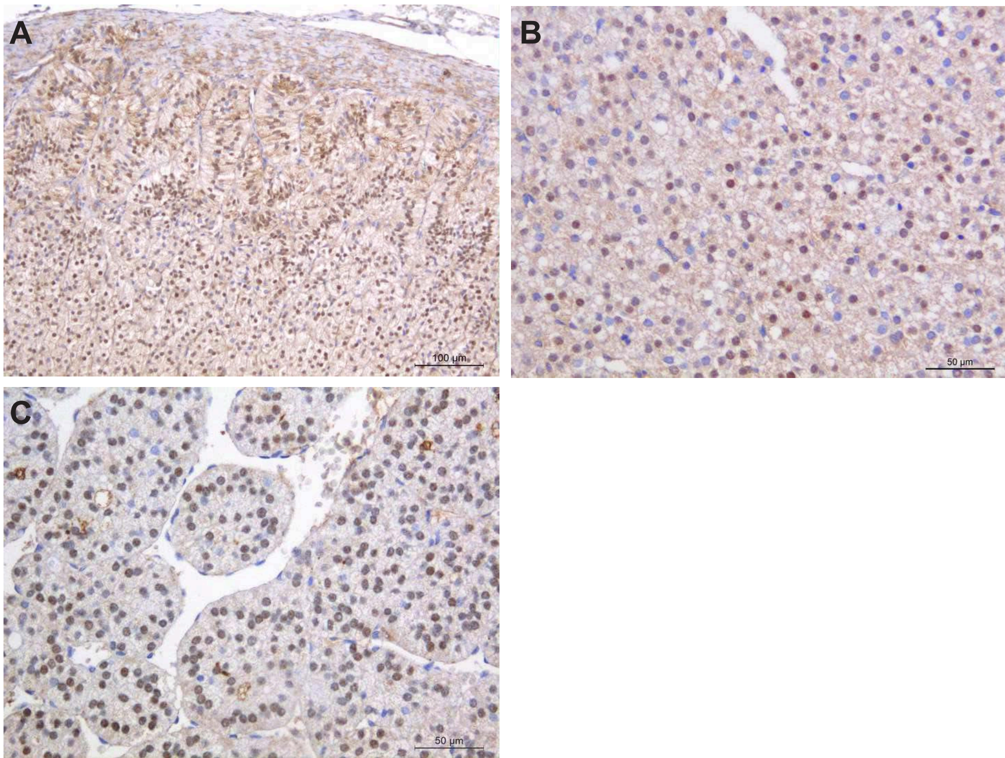

Figure 1

Representative examples of immunohistochemical expression of steroidogenic factor 1 (SF-1) in zona glomerulosa and zona fasciculata of a normal canine adrenal gland (A) and cortisol-secreting adrenocortical adenoma (B) and carcinoma (C). Scale bars represent 100 µm (A) and 50 µm (B and C).

Results

Relative mRNA expression abundances of SF-1 in normal adrenal glands did not differ from those in adenomas and carcinomas. SF-1 protein was expressed in all normal adrenals and ATs. IHC demonstrated predominantly nuclear staining in normal adrenals, with the strongest intensity in the zona glomerulosa and zona fasciculata (Figure 1A). In the majority of adrenocortical adenomas and carcinomas the SF-1 positive cells showed nuclear staining and occasional a cytoplasmic immunoreactivity was observed (Figure 1B and 1C). All 15 dogs from which follow up information was available were diagnosed with carcinoma. In a group of dogs with short survival, 7 dogs had reoccurrence of ACTH-independent hypercortisolism from 3 to 24 mo after surgery. Besides the physical and biochemical changes associated with hypercortisolism, liver and/or lung metastasis were detected in six of these dogs at the time of evaluation. In 1 dog regrowth of the AT was seen by

ultrasonography. In 8 of dogs in remission for at least 2.5 yr after surgery, 2 dogs are still alive (54 and 60 mo post adrenalectomy), and 5 dogs were euthanized due to reasons unrelated to hypercortisolism (range 45-72 mo post adrenalectomy). In 1 dog there was a reoccurrence of hypercortisolism due to the cortisol-secreting AT in a contralateral adrenal gland (35 mo post adrenalectomy) and the dog has been treated medically with trilostane. The relative mRNA expression abundances of SF-1 were greater (2.46-fold, P = 0.020) in the group with recurrence of hypercortisolism within 2.5 yr than in the group of dogs that were in remission at least 2.5 yr after surgery.

Discussion

Increasing evidence of the importance of SF-1 in adrenocortical tumorigenesis in humans and mice motivate the relevance to study its expression in canine ATs [8,9,10]. In the present study, the relative SF-1 mRNA expression in normal adrenals and cortisol-secreting ATs does not differ and yet high SF-1 expression is associated with poor clinical outcome. At a glance, this may seem contradictory, however, it could be related to the functional role of SF-1, which clearly depends on the cellular context [2,6]. In the normal adult differentiated adrenocortical cell, the major role of SF-1 is the regulation of steroidogenesis. In fetal adrenal development, i.e., non-differentiated cells, SF-1 stimulates adrenal growth, independent of steroid synthesis [16,17]. Hypothetically, carcinomas might dedifferentiate towards a more fetal phenotype. Apart from expression also activation by yet unknown mechanisms may play a role [18]. Another possible explanation could lie in the fact that whole tissue explants of normal adrenals were used as a control. It is possible, that the results would have been different when AT tissue would be compared to zona fasciculata only.

In the group of ATs with poor outcome, i.e., the recurrence of hypercortisolism within 2.5 yr after surgery, and high relative SF-1 expression, metastases were present in the majority of dogs. In one dog, the recurrence of hypercortisolism was associated with AT in the contralateral adrenal gland 35 mo after surgery. The possibility, that this AT was the metastasis of the previously removed AT seems unlikely, although it could not be completely excluded [13,19]. Bilateral adrenal gland involvement in this case is also possible. Angiogenesis is paramount in tumor development and metastasis, with angiopoietin 2 (ANGPT2) being a key player. Using a ChIP-on-chip approach, a SF-1-binding region was identified in the human *ANGPT2* promoter, and SF-1-dependent activation of ANGPT2 transcription was confirmed in luciferase assays [20]. This provides evidence of ANGPT2 as an

important target of SF-1 in the adrenal gland and suggests that the regulation of angiogenesis in ATs might be related to SF-1 expression. Recent research demonstrated elevated abundances of ANGPT2 in canine cortisol-secreting ATs [21], and studies about the link between ANGPT2 and SF-1 are warranted.

Immunohistochemistry demonstrated a predominantly nuclear pattern of SF-1 staining. This is in agreement with the data in other species. The occasional cytoplasmic staining observed, could be ascribed to the use of a polyclonal antibody [22]. In human adrenocortical carcinoma, there is a negative association between the SF-1 staining intensity and survival, which resulted in the inclusion of SF-1 IHC among the prognostic factors [10]. In the present study, the low number of dogs available for follow up was a limiting factor to objectively assess the prognostic value of SF-1 staining. A multicenter approach including large numbers of dogs with cortisol-secreting ATs should be set up in the future to test the utility of SF-1 mRNA and/or protein expression as a prognostic marker.

Recently, drugs targeting SF-1 activity have been developed. Isoquinolinone compounds, so called SF-1 inverse agonists, inhibit the constitutive transcriptional activity of SF-1. They have been shown to selectively inhibit cell proliferation in a human adrenocortical cell line, and to inhibit steroid hormone secretion [23,24]. Based on the results of the present study, drugs targeting SF-1 may be beneficial in medical management of at least a subgroup of canine ATs. In vitro studies on primary canine AT cell cultures are warranted to evaluate their effect on steroidogenesis and proliferation and disclose their therapeutic potential.

In conclusion, our study demonstrated SF-1 expression in normal adrenals and cortisol-secreting ATs and suggests that SF-1 might possess a prognostic value and could be an attractive target for a medical approach.

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Chapter 6

Expression of angiogenesis-related genes in canine cortisol-secreting adrenocortical tumors

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Abstract

The aim of this study was to evaluate the expression of angiogenesis-related genes in canine cortisol-secreting adrenocortical tumors (ATs). Quantitative RT-PCR analysis revealed mRNA encoding for vascular endothelial growth factor (*VEGF*), VEGF-receptors 1 and 2, angiopoietin 1 and 2 (*ANGPT1* and *ANGPT2*), the splice variant $ANGPT2_{443}$, the ANGPT-receptor *Tie2*, and basic fibroblast growth factor (*bFGF*) in 38 canine cortisol-secreting ATs (26 carcinomas and 12 adenomas) and 15 normal adrenals. The relative expression of both *ANGPT2* and $ANGPT2_{443}$ was higher in adenomas (P = 0.020 for *ANGPT2* and P = 0.002 for $ANGPT2_{443}$) and carcinomas (P = 0.003 for *ANGPT2* and $P < 0.001$ for $ANGPT2_{443}$) compared to normal adrenals, and this enhanced expression was also detected using Western blot. Immunohistochemistry demonstrated expression of ANGPT2 protein in AT cells and in vascular endothelial cells of carcinomas, while Tie2 was mainly present in the tumor vascular endothelial cells. The *ANGPT2*/*ANGTPT1* ratio, a marker for a pro-angiogenic state, was higher in both adenomas (P = 0.020) and carcinomas (P = 0.043). Using the human H295R cortisol-producing adrenocortical carcinoma cell line, we were able to demonstrate that the *ANGPT2* expression was stimulated by cAMP and progesterone, but not by cortisol. In conclusion, canine cortisol-secreting ATs have enhanced ANGPT2 expression with a concomitant shift towards a pro-angiogenic state. Based on this information, treatment modalities may be developed that interfere with ANGPT2 expression, including inhibition of the cAMP/PKA pathway, or of the effect of ANGPT2, by using specific ANGPT2 inhibitors.

Introduction

Adrenocorticotropin (ACTH)-independent hypercortisolism in dogs as a result of autonomous glucocorticoid production by an adrenocortical tumor (AT) accounts for about 15% of cases of spontaneous canine hypercortisolism [1]. The treatment of choice is adrenalectomy, because the successful complete removal of the affected adrenal gland will eliminate the clinical signs related to glucocorticoid excess without the need for lifelong medication. However, invasive growth in the surrounding tissues and/or metastasis may preclude complete removal. Factors involved in tumor growth and metastasis of canine cortisol-secreting ATs are largely unknown.

In general, angiogenesis, the process of new blood vessel formation from existing vasculature, is an important factor in tumor development and metastasis. By means of intra-tumoral angiogenic feedback loops, tumors may activate angiogenesis and provide themselves with the nutrients and oxygen necessary to grow beyond a certain size [2]. In human cancer research, the use of anti-angiogenic drugs is one of the most rapidly emerging therapeutic strategies [3]. The aim of the present study was to evaluate the expression of angiogenesis-related genes in canine cortisol-secreting ATs. Knowledge on the role of angiogenesis in AT development may help in the development of new treatment modalities.

In the regulation of tumor angiogenesis and the development of anti-angiogenic drugs, basic fibroblast growth factor (bFGF), the vascular endothelial growth factor (VEGF) family and the angiopoietin (ANGPT) family play a pivotal role [2,4]. Although inhibition of bFGF is still in the early stages of development, its role as a potent mitogen for fetal and adult adrenocortical cells [5,6], and its increased expression in human adrenal medullary tumors [7] make bFGF a target of specific interest in adrenal gland pathology.

Drugs targeting VEGF have shown potential in various clinical and pre-clinical trials, inhibiting angiogenesis and tumor growth [2,8]. Furthermore, high VEGF expression has been associated with increased microvascular density in human adrenocortical carcinoma [9].

Angiopoietin signaling has likewise been implicated as a significant factor in the pathogenesis of human ATs [10]. Especially, the ratio between ANGPT1 and ANGPT2 is considered an important indicator of activation of the angiogenic switch in tumors [11]. Selective ANGPT2 inhibition, either combined with VEGF-inhibition or by itself, has shown promise in slowing tumor angiogenesis and tumor growth in different

tumor types [12,13]. The expression of ANGPT2 in the human adrenal gland is thought to be regulated by ACTH-cAMP-Steroidogenic Factor 1 (SF-1) signaling [14,15]. Recently, we demonstrated that a large proportion of canine cortisol-secreting ATs harbors an activating mutation in GNAS, the gene responsible for cAMP production upon ACTH-stimulation [16]. Data about the relation between cAMP signaling and angiogenesis in canine ATs are lacking.

The aim of the study was to evaluate the expression of bFGF, VEGF, VEGF-receptors 1 and 2, ANGPT1 and ANGPT2, the splice variant $ANGPT2_{443}$, and the ANGPT-receptor Tie2 in canine cortisol-secreting carcinomas and adenomas, compared with that in normal adrenals. In addition, we investigated whether the expression of genes of interest was influenced by cAMP or the adrenocortical hormones cortisol and progesterone *in vitro*.

Materials and methods

Patient material

In this study 38 canine cortisol-producing ATs and 15 normal adrenal glands were used. Adrenal glands from healthy dogs were available as archived tissue for comparison with AT tissue obtained from patients. After surgical removal of an AT in the patients or resection of a normal adrenal gland in the healthy dogs, the tissue was stored on ice, inspected, and material was saved for quantitative RT-PCR (qPCR) analysis and histopathology. The fragments for RNA isolation were cut and snap frozen in liquid nitrogen within 10 min. They were kept at -80°C until further use. The remaining part of the AT tissue was immersed in formalin for fixation and embedded in paraffin after 24 h to 48 h. The tumor group consisted of all histologically confirmed ATs derived from patients referred to the Department of Clinical Sciences of Companion Animals of the Faculty of Veterinary Medicine in Utrecht between 2001 and 2009 with clinical signs of hypercortisolism. The diagnosis of ACTH-independent Cushing's syndrome due to an AT was based upon (i) elevated urinary corticoid-to-creatinine ratios, which were not suppressible with high doses of dexamethasone, (ii) suppressed or even undetectable plasma ACTH concentrations [1] and (iii) demonstration of an AT by ultrasonography and/or computed tomography [17]. All dogs underwent unilateral adrenalectomy. The dogs' ages at the time of surgery ranged from 6 to 14 yr (mean, 9 yr). Twelve dogs were mongrels and the other dogs were of 10 different breeds. Eighteen dogs were male (eight castrated) and 20 female (15 neutered). The ages of the control dogs ranged

from 2 to 5 yr. Five of the control dogs were male and 10 were female, all control dogs were intact. Permission to use the AT tissue for this study was obtained from all patient owners and the study was approved by the Ethical Committee of Utrecht University.

PCR primers (Position)		Sequence (5'-3')	T_m	Product length (bp)
cf_ANGPT1	*Fw*	AAT AAT ATG CCA GAA CCC AAA AAG	62°C	162
(926 -1087)	*Rv*	CCC CAG CCA ATA TTC ACC AGA G		
cf_ANGPT2	*Fw*	ACA GCA TCG GGA GAA GGC AGT ATC	54°C	553 (FL)
(106-658)	*Rv*	TCT TCT TTT ATT GAC CGT AGT TGA		398 ($_{443}$)
cf_ANGPT2-FL	*Fw*	AGA ACC AGA CTG CCG TGA T	65°C	110
(376-485)	*Rv*	TGT TGT CTG ATT TAA TAC TTG TGC		
cf_ANGPT2$_{443}$	*Fw*	TAC GCA GTG GCT AAT TAA GGT ATT	64,5°C	226
(293-518)	*Rv*	CTG GAG CTG ATC TTT CTC TTC TTT		
cf_Tie2	*Fw*	CAG CTT ACC AGG TGG ACA TTT TTG	58°C	104
(25-138)	*Rv*	GTC CGC TGG TGC TTG AGA TTT AG		
cf_VEGF	*Fw*	CTT TCT GCT CTC CTG GGT GC	58°C	102
(6-107)	*Rv*	GGT TTG TGC TCT CCT CCT GC		
cf_VEGFR1	*Fw*	GGC TCA GGC AAA CCA CAC	63°C	190
(189-378)	*Rv*	CCG GCA GGG GAT GAC GAT		
cf_VEGFR2	*Fw*	GGA AGA GGA AGT GTG TGA CCC C	64°C	181
(3606-3785)	*Rv*	GAC CAT ACC ACT GTC CGT CTG G		
cf_bFGF	*Fw*	TTC TTC CTG CGG ATC CA	61°C	76
(2251-2326)	*Rv*	GTT GCA ATT TGA CGT GGG		
hs_ANGPT2	*Fw*	AAC ATC CCA GTC CAC CTG AG	60°C	102
(1820-2021)	*Rv*	GGT CTT GCT TTG GTC CGT TA		
hs_TBP	*Fw*	TGC ACA GGA GCC AAG AGT GAA	63,5°C	132
(940-1071)	*Rv*	CAC ATC ACA GCT CCC CAC CA		
hs_RPS19	*Fw*	CCT TCC TCA AAA AGT CTG GG	61°C	95
(431-525)	*Rv*	GTT CTC ATC GTA GGG AGC AAG		

Table 1

PCR primer pairs used for amplification in human and canine samples of canine Angiopoietin (ANGPT) 1 and 2, ANGPT2 full length (FL) and ANGPT2$_{443}$, Tie2, Vascular endothelial growth factor (VEGF), VEGF receptor (VEGFR) 1 and 2, basic fibroblast growth factor (bFGF) and human ANGPT2, Tata binding protein (TBP) and ribosomal protein S19 (RPS19). All positions are based on the mRNA sequence, as published on the NCBI GenBank database. Accession numbers used: canine ANGPT1: NM_001005754, ANGPT2: NM_001048126.1, Tie2: AF282848, VEGF: NM_001003175.2, VEGFR1: XM_534520, VEGFR2: NM_001048024.1, bFGF: XM_533298.1. Human ANGPT2: NM_001147.2, TBP: NM_003194.4, RPS19: NM_001022.3 Abbreviations: cf: Canis familiaris, hs: Homo sapiens

Histopathology

Histopathological evaluation was performed on formalin fixed and paraffin embedded tissue slides of all samples and used to confirm the diagnosis and to classify the tumors. All histological evaluations were performed by a single pathologist. Classification was performed based on the criteria described by Labelle *et al*. [18]. Classification as a carcinoma was based on histological evidence of vascular invasion, peripheral fibrosis, capsular invasion, trabecular growth, hemorrhage, necrosis, and single cell necrosis. Typical histological characteristics of adenomas were hematopoiesis, fibrin thrombi, and cytoplasmic vacuolization. Based on these criteria, the tumor group consisted of 12 adenomas and 26 carcinomas.

Total RNA extraction and reverse transcription

Total RNA for qPCR analysis was isolated from tissue and cell culture samples using the RNeasy mini kit (Quiagen, Hilden, Germany), according to manufacturer's protocols. An optional DNAse step was performed to avoid DNA contamination. RNA concentrations were measured on the NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE). Synthesis of cDNA was performed using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA), according to manufacturer's protocols. For all samples, one cDNA reaction was performed without Reverse Transcriptase (RT-), to check for contamination with genomic DNA.

Quantitative RT-PCR

Quantitative RT-PCR primers for all target genes were designed using DNA-star primer select v8.1, Oligo-explorer v1.1.0 and/or Perl-primer v1.1.14 according to the parameters in the Bio-Rad iCycler manual, and ordered from Eurogentec (Maastricht, The Netherlands). Primers for distinguishing both *ANGPT2* variants were designed to anneal to areas of the transcript unique to each isoform. For each primer pair a qPCR temperature gradient was performed, to determine the optimal annealing temperature. Formation of the proper PCR products was confirmed by a sequencing reaction, using the ABI3130XL Genetic analyzer (AB applied Biosystems, Carlsbad, CA) according to manufacturer's protocol.

A pool of all cDNA samples was used to create a four-fold dilution series for use as a standard. The rest of the cDNA was diluted four times with milliQ water, to achieve a working stock. On all of the canine samples, mRNA expression levels for the following target genes were measured: *VEGF*, *VEGFR1*, *VEGFR2*, *ANGPT1*, *ANGPT2* (both full length (FL) and the splice variant $ANGPT2_{443}$), *Tie2* and *bFGF*. To correct

for differences in cDNA concentration, ribosomal protein S5 (*RPS5*), *RPS19* and hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) were used as reference genes [19]. On the human H295R samples mRNA expression levels were determined for *ANGPT2*. Tata-binding protein (*TBP*) and *RPS19* were used as reference genes. Analysis of the relative expression levels of the reference genes revealed no significant differences between groups and their expression was shown to be stable using GeNorm software [20], justifying their use as reference genes.

All qPCR reactions were performed on a MyIQ single color real-time PCR detection system (BioRad). Detection was performed using SYBRgreen supermix (BioRad) and data were analyzed using iQ5 software (BioRad). The raw data were used to calculate the reaction efficiency. Reaction efficiencies between 90% and 110% were accepted. Calculation of normalized relative expression levels for each of the target genes was performed using the ΔΔ-Ct method [21].

Human and canine PCR primers and their characteristics are listed in Table 1.

Western blot

To evaluate whether the differences on mRNA level for ANGPT2 were also reflected at the protein level, Western blot for ANGPT2 was performed on all tissue samples. Protein was isolated from the frozen tissue samples using radioimmunoprecipitation assay (RIPA) buffer. Protein concentrations were measured using the DC protein assay (500-0116, BioRad). Gel electrophoresis was performed on 7.5% polyacrylamide gels together with a dual color Precision plus Protein Standard (BioRad) for molecular weight determination. After gel electrophoresis, proteins were transferred onto Hybond ECL nitrocellulose membrane (Amersham, GE Healthcare, Diegem, Belgium). Nonspecific binding was blocked using 4% ECL in a buffer of 50 mM Tris (pH 7.6), 150 mM NaCl and 0.1% Tween (0.1% TBST). Blots were incubated overnight at 4°C using a goat polyclonal anti-ANGPT2 antibody known to cross-react with canine ANGPT2 (C-19, sc-7015, Santa Cruz biotechnologies, Santa Cruz, CA, USA), in a dilution of 1:200. Blots were washed and incubated for 1 h at room temperature with a secondary antibody (donkey anti-goat HRP conjugated IgG, sc-2020, Santa Cruz) at a dilution of 1:20,000. Proteins were visualized using the ECL advanced Western blotting detection kit (Amersham RPN2135, GE Healthcare) and measurement of chemiluminescence (ChemiDoc XRS, BioRad). As a loading control all blots were stripped and incubated with α-tubulin antibody (TUB-1A2, ab11325, AbCam, Cambridge, UK) in a 1:2,000 dilution. An HRP-conjugated anti-mouse IgG (R&D Systems, Minneapolis, MN, USA), was used as secondary antibody in a dilution of 1:20,000. Protein expression was quantified

using QuantityOne software (BioRad) and normalized to the intensity of α-Tubulin staining. The specificity of the ANGPT2 staining was confirmed by pre-incubation of the primary antibody with a blocking peptide for ANGPT2 (sc-7015P, Santa Cruz biotechnologies) for 2 h at room temperature in a 1:40 dilution.

Immunohistochemistry

Tissue localization of ANGPT2 and its receptor Tie2 was evaluated in a representative subset of tumors and normal adrenals. For this purpose, immunohistochemical staining was performed on 21 formalin-fixed, paraffin embedded tissue slides (10 carcinomas, seven adenomas and four normal adrenals) for ANGPT2 and on nine tissue slides (three carcinomas, three adenomas and three normal adrenals) for Tie2. Tissue slides were rehydrated in a series of xylene-alcohol baths. Antigen retrieval for ANGPT2 staining was performed using 10 mM sodium citrate buffer pH 6, for 20 min at 95°C. For Tie2 staining, no antigen retrieval was used. To block endogenous peroxidase activity, slides were incubated with peroxidase block (S2001, Dako, Glostrup Denmark) for 5 min (ANGPT2) or 20 min (Tie2). Nonspecific binding sites were blocked for 20 min, using 10% normal goat serum in PBS (ANGPT2) or 5% BSA in PBS (Tie2). A rabbit-anti-human anti ANGPT2 antibody (Ab6583, AbCam) was used, in a 1:250 dilution in 10% normal goat serum in PBS. For Tie2, a rabbit anti-human antibody (sc-9026, Santa Cruz Biotechnologies) was used in a 1:200 dilution in 1% BSA in PBS. Slides were incubated with the primary antibody for 120 min at room temperature (ANGPT2) or overnight at 4°C (Tie2). Subsequently, all slides were incubated with Anti-rabbit HRP conjugated secondary antibody (Dako K4003) for 30 min. Antibody detection was performed using Dako K3468 HRP substrate. All slides were incubated with DAB for 4 min (ANGPT2) or 1.5 min (Tie2) and subsequently counterstained with heamatoxylin. The specificity of the reaction for ANGPT2 was confirmed by using blocking peptide (Angiopoietin 2 peptide, ab98299, AbCam) in a concentration of 100 μg/mL. Pre-incubation of the antibody with this blocking peptide abolished all staining. For Tie2, the specificity of the reaction was verified by omission of the primary antibody as well as by using normal rabbit serum as first antibody. As a positive control, human placenta and canine ovarian tissue slides were used. All slides were analyzed in a descriptive manner, to determine the presence of positive staining, localization within the tissue, and cellular localization.

Cell culture

To investigate whether ANGPT expression was influenced by the cAMP/PKA pathway, *in vitro* experiments were performed using the human H295R adrenocortical carcinoma cell line. To exclude that the increase in *ANGPT2* was due

to an increase in cAMP-induced cortisol or progesterone secretion, the effect of both dexamethasone and progesterone on *ANGPT2* mRNA expression was investigated. The H295R cells were cultured according to a protocol described previously [22], adapted for use with commercially available Nu-serum. Growth medium consisted of DMEM/ F12 (1:1) (GibcoBRL, Breda, The Netherlands), supplemented with 1% (v/v) insulin, transferrin, and selenium (ITS) supplement (GibcoBRL); 1.25% (w/v) BSA, (Sigma-Aldrich, St Louis, MO), 1% (w/v) penicillin/streptomycin and 2.5% (v/v) Nu-serum (BD biosciences, Franklin lakes, NJ). For all experiments, cells were plated onto 24-wells Primaria culture plates at a density of $5x10^5$ cells per well and allowed to attach for 24 h. Next, cells were incubated with 8-bromo-cAMP sodium salt (Sigma-Aldrich, final concentrations in the medium: 10^{-3}-10^{-5} M), progesterone (Sigma-Aldrich, 2×10^{-8} M) or dexamethasone (Sigma-Aldrich, 10^{-7} M) in serum free medium, using a physiologic range of concentrations. After 15 min Nu-serum was added to a concentration of 2.5%. After incubation for 2 to 48 h the cells were prepared for RNA isolation and medium was collected for cortisol measurement. All stimulations and controls were performed in replicates of four, and for each time point an unstimulated control was included. Cortisol concentrations in the culture media were measured by means of radioimmunoassay (RIA), as described previously [23].

Statistical analyses

Statistical analyses were performed using SPSS16 (IBM, Armonk, NY). Individual relative mRNA expression levels for all qPCR experiments were calculated using the ΔΔ-Ct method [21]. The relative expression levels as obtained by qPCR, the normalized protein concentrations as obtained by Western blot, and the cortisol concentrations from cell culture stimulation experiments were analyzed using one-way ANOVA and Scheffe's post-hoc test or Students t-test, in order to establish the presence of differences between the groups and its statistical significance. For Western blot and qPCR experiments on canine tissue, the relative expression levels in normal adrenals were compared with those in adenomas and carcinomas, and between adenomas and carcinomas. For all H295R stimulation experiments, relative expression levels and cortisol concentrations of treated sample groups were compared with the values in the untreated control group at the same time-point. Correlations between the relative expression levels of *ANGPT2-FL* and $ANGPT2_{443}$ were calculated using Pearson's correlation coefficient. For all statistical tests, a *P*-value below 0.05 was considered significant.

Results

Canine cortisol-secreting ATs overexpress ANGPT2 and have an increased ANGPT2/ANGPT1 ratio

Analysis of the relative mRNA expression levels of the target genes revealed no significant differences in the expression levels of *ANGPT1*, *Tie2*, *VEGF*, *VEGFR2* and *bFGF*. A significantly lower expression of *VEGFR1* was found in adenomas when compared to normal adrenals (0.34-fold, $P = 0.006$).

Using variant-specific primers, the presence of the ANGPT2 splice variant $ANGPT2_{443}$ was demonstrated for the first time in canine ATs. The relative expression levels of *ANGPT2-FL* were significantly higher in the tumor groups when compared to normal adrenals: 2.73-fold higher ($P = 0.020$) in adenomas and 4.56-fold higher ($P = 0.003$) in carcinomas. The increase in relative expression of $ANGPT2_{443}$ was 3.18-fold ($P = 0.002$) in adenomas and 6.60-fold ($P < 0.001$) in carcinomas. For $ANGPT2_{443}$, the difference in relative expression between adenomas and carcinomas was also significant (2.07-fold, $P = 0.036$).

The *ANGPT2* to *ANGPT1* ratio was calculated for adenomas, carcinomas and normal adrenals, and the ratio was significantly higher in both the adenomas (3.96-fold, $P = 0.020$) and carcinomas (7.82-fold, $P = 0.043$) compared to normal adrenals (Fig. 1A, B). Levels of *ANGPT2-FL* and $ANGPT2_{443}$ correlated significantly in both normal adrenals and ATs (r = 0.96; $P < 0.001$).

The presence and expression of ANGPT2 protein in canine ATs and normal adrenals was studied by Western blotting. In most of the samples two bands were detected at 61 and 68 kDa, corresponding to the expected weights of $ANGPT2_{443}$ and ANGPT2-FL, respectively. Both ANGPT2-FL and $ANGPT2_{443}$ showed a significantly more intense staining ($P = 0.04$) in the ATs when compared to normal adrenal tissue (Fig. 1C, D). No significant difference between adenomas and carcinomas could be detected.

Twelve patients were available for follow-up: eight of them developed symptoms and signs of hypercortisolism within 2 yr after surgery and the recurrence of hypercortisolism was confirmed by endocrine testing. Four of them were in remission for at least 2 yr after adrenalectomy. The relative mRNA expression levels of *ANGPT2-FL* and $ANGPT2_{443}$ compared to normal adrenals were higher in the group with recurrence when compared to those without recurrence of hypercortisolism (6.90-fold vs. 2.57-fold for *ANGPT2-FL* and 10.11-fold vs. 3.37-fold for $ANGPT2_{443}$). However, these differences did not reach a significant level.

Immunohistochemical staining for ANGPT2 is present in both tumor cells and vascular endothelial cells in canine ATs, and virtually absent in normal adrenals

Immunohistochemical staining of 17 canine cortisol-secreting ATs (seven adenomas and 10 carcinomas) and four normal adrenals revealed a striking difference in ANGPT2 staining between normal adrenals and ATs. In normal adrenal cortices, no

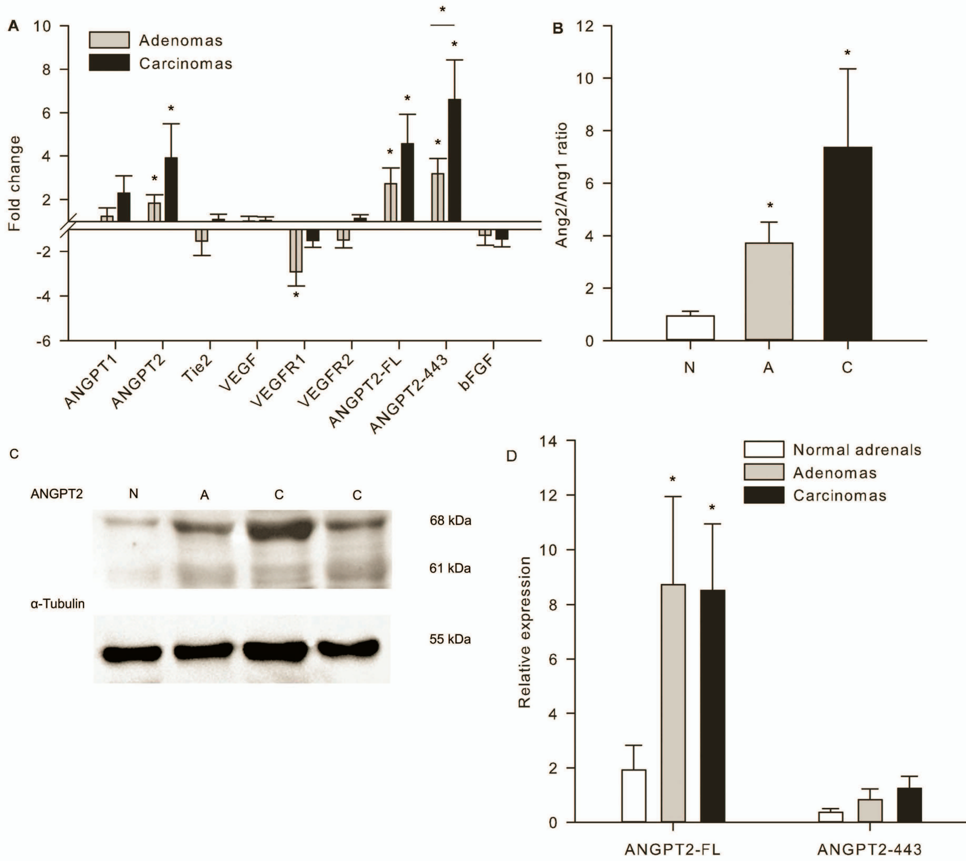

Figure 1

Relative expression levels of target genes and proteins in 38 canine ATs and 15 normal adrenal glands. Relative mRNA expression of target genes in canine cortisol-secreting adenomas (A) and carcinomas (C) when compared with normal adrenal glands (N), as measured by qPCR and calculated using the 2^-ΔΔCt method. (A) ANGPT2/ANGPT1 ratios in N, A and C. (B) Representative example of Western Blot results, showing ANGPT2 full length (FL) (68 kDa) and ANGPT2$_{443}$ (61 kDa) bands in N, A and C and corresponding Tubulin bands (55 kDa). (C) Graph representing the protein expression levels of both isoforms of ANGPT2 in N, A, and C samples. (D) Bars represent mean +/- SEM. Significant changes ($P < 0.05$) are marked with an asterisk. Abbreviations: ANGPT: Angiopoietin, FL: Full length variant, bFGF: basic Fibroblast Growth Factor, VEGF: Vascular Endothelial Growth Factor, VEGFR: VEGF receptor

staining was detected in the zona reticularis (ZR) and zona fasciculata (ZF). The zona glomerulosa (ZG) exhibited very low to absent staining (Fig. 2C). In all ATs a weak to moderate cytoplasmic granular staining was present in most of the tumor cells, while islands of moderately to strongly stained tumor cells were scattered throughout the tumor slides (Fig. 2D and E). Additionally, in part of the ATs a strong cytoplasmic

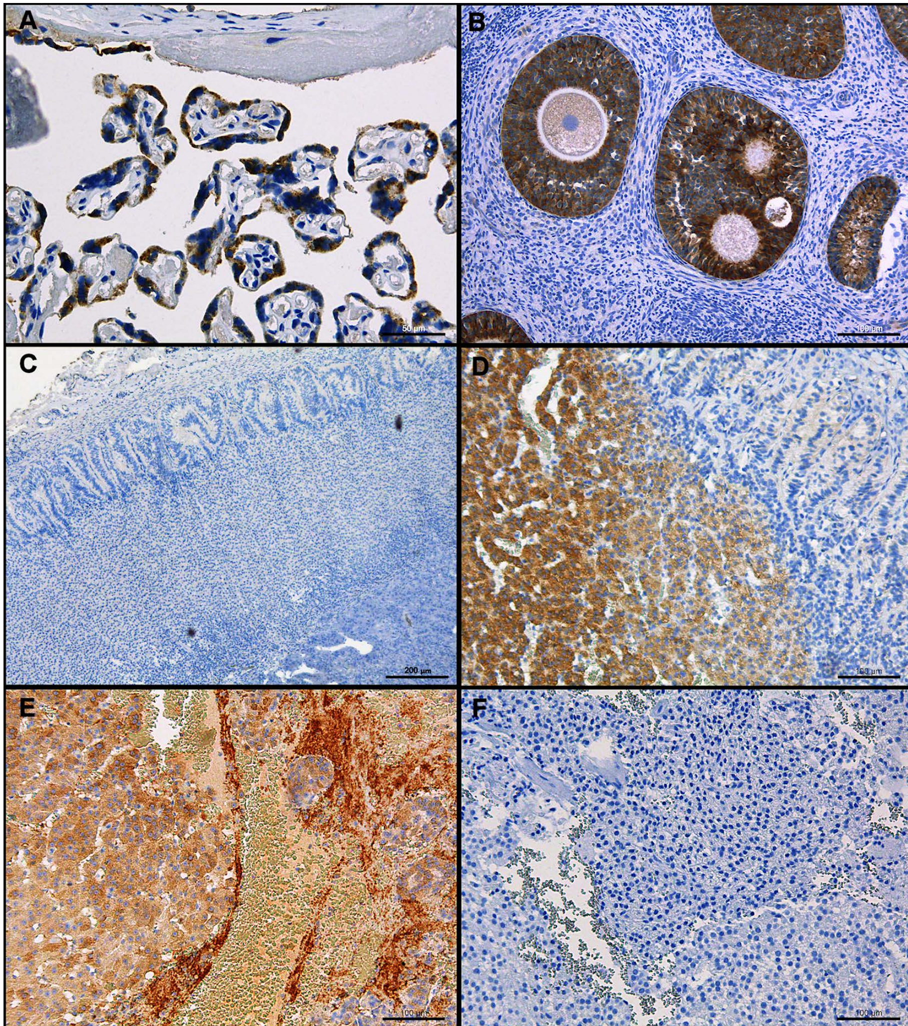

Figure 2

Immunohistochemical staining for Angiopoietin 2 in 17 ATs and 4 normal adrenal glands. Representative examples of immunohistochemical staining for Angiopoietin 2 in human placenta (human positive control tissue) (A), canine ovary (canine positive control tissue) (B), normal canine adrenal cortex (C), tumor cell staining in canine cortisol-secreting adrenocortical adenoma (D), tumor cell and endothelial staining in canine cortisol-secreting adrenocortical carcinoma (E), and in the same carcinoma with antibody pre-incubated with blocking peptide (F).

Bars represent 50 µm (A), 100 µm (B and D-F) and 200 µm (C) respectively.

granular staining was seen in the vascular endothelial lining (Fig. 2E). The endothelial staining was detected in only one of the seven adenomas and in six of the 10 carcinomas.

Immunohistochemical staining for the Tie2 receptor, the presence of which is needed to enable an effect of ANGPT2, was performed in six cortisol-secreting ATs (three adenomas and three carcinomas) and three normal adrenals. Very low to negative staining was seen in the normal adrenal cortex and in most AT cells (Fig. 3A and B). In contrast, the tumor vasculature showed a clear cytoplasmic granular staining pattern of the vascular endothelial cells which could not be found in normal adrenal cortex (Fig. 3D). Additionally, in some ATs distinct regions of Tie2 positive tumor cells were detected, with a cytoplasmic granular staining pattern (Fig. 3D).

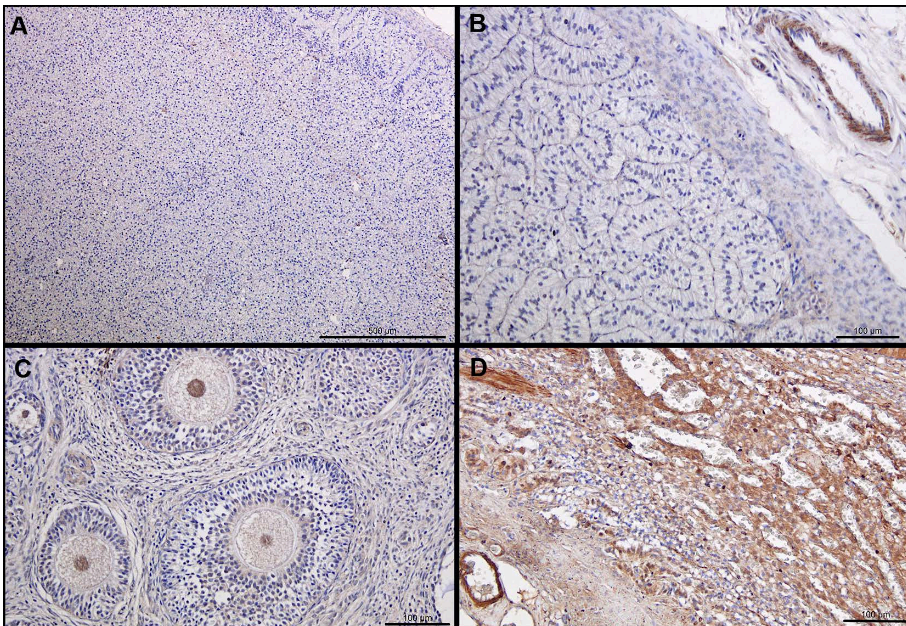

Figure 3

Immunohistochemical staining for Tie2 in 6 ATs and 3 normal adrenal glands. Representative examples of immunohistochemical staining for Angiopoietin receptor Tie2 in normal canine adrenal cortex (overview) (A), normal canine adrenal cortex (detail, including blood vessel) (B) canine ovary (positive control tissue) (C) and canine cortisol-secreting adrenocortical carcinoma showing extensive vasculature, Tie2 positive vascular endothelial cells and focal areas of positive tumor cells (D).
Bars represent 500 µm (A) or 100 µm (B-D), respectively.

Cyclic AMP stimulates ANGPT2 mRNA expression in H295R cells

As cortisol secretion in adrenocortical cells is cAMP-dependent, stimulation of H295R cells using 8-bromo-cAMP resulted in a dose-dependent increase of cortisol in the medium. Interestingly, the highest 8-bromo-cAMP concentration also clearly stimulated the mRNA expression of *ANGPT2* during the first 8 h of incubation (3.25-fold, P = 0.026), while expression returned to baseline values after 48 h (Fig. 4). Dexamethasone did not influence the expression of *ANGPT2*, but progesterone led to a transient induction of *ANGPT2* expression (1.78-fold, P = 0.037) (Fig. 5).

Discussion

Angiogenesis is recognized as an important factor in tumor development and metastasis. In the present study we investigated the hypothesis that the highly vascularized, cortisol-secreting ATs have a higher expression of certain angiogenesis-related genes than normal adrenals. The results of the present study show a markedly increased relative expression of ANGPT2, both at the mRNA and protein level, in canine cortisol-secreting adrenocortical adenomas and carcinomas compared to normal adrenals.

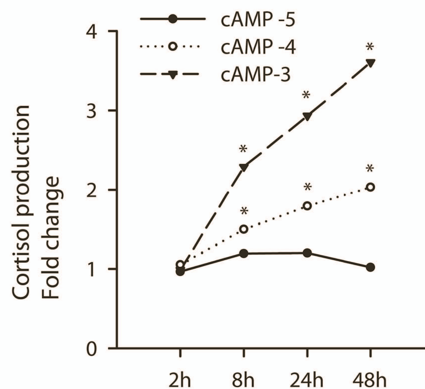

Figure 4

Effect of 8-bromo-cAMP stimulation of H295R cells on cortisol production. Dose-dependent stimulation of cortisol production in human H295R cells as a function of time after stimulation with 8-bromo-cAMP. Cortisol production is depicted as a fold change towards the production in non-stimulated cells cultured under the same conditions and for the same period of time. Significant changes (P < 0.05) are marked with an asterisk. Legend: cAMP -5, -4 and -3: stimulation with 8-bromo-cAMP in respectively 10^{-5}, 10^{-4} and 10^{-3} M

The concurrently increased *ANGPT2*-to-*ANGPT1* ratio is a reliable indicator for a shift of the angiogenic balance towards a pro-angiogenic state [11]. These results are in line with the findings by Giordano et al., who reported a 3-fold increase of ANGPT2 expression in human adrenocortical carcinomas using microarray.

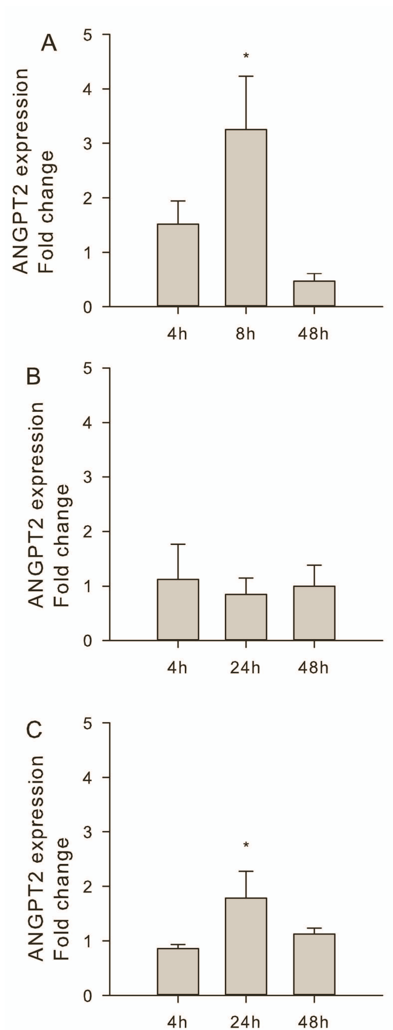

Figure 5

Relative mRNA expression of ANGPT2 upon stimulation of H295R cells. Effects of stimulation with 8-bromo-cAMP (A, 10^{-3}M), dexamethasone (B, 10^{-7}M) and progesterone (C, 2.10^{-8} M) on mRNA expression levels of ANGPT2 as a function of time in H295R human adrenocortical carcinoma cells. Effect on relative expression levels is depicted as a fold change towards the expression in non-stimulated cells cultured under the same culture conditions and for the same period of time. Significant changes ($P < 0.05$) are marked with an asterisk.

Based on their observations, Giordano *et al*. suggested that ANGPT-induced angiogenesis is an important aspect of human adrenocortical tumorigenesis [10].

This is the first study demonstrating the presence of the ANGPT2 splice variant $ANGPT2_{443}$ in adrenocortical tumor tissue. Moreover, the fold changes in the relative mRNA expression of $ANGPT2_{443}$ even exceeded that of *ANGPT2-FL* when ATs were compared with normal adrenocortical tissue. Although no data have been published on the presence and regulation of $ANGPT2_{443}$ in human adrenocortical tissue, expression analyses of $ANGPT2_{443}$ in other human tumor cell lines and primary tumor tissues indicate a role in tumorigenesis for this splice variant [24]. The increased relative expression of $ANGPT2_{443}$ mRNA in ATs, as well as its higher expression in malignant tumors suggest a tumor-specific role for this variant in the development of canine ATs.

The higher expression of *ANGPT-FL* and $ANGPT2_{443}$ and the higher *ANGPT2*-to-*ANGPT1* ratio in carcinomas compared to adenomas suggest involvement of the ANGPT family in adrenocortical carcinogenesis. This is supported by the higher *ANGPT2-FL* and $ANGPT2_{443}$ expression levels in the group of dogs showing recurrence of hypercortisolism within a 2-year-period, although this difference did not reach significance, possibly due to low numbers. The higher expression of *ANGPT2-FL* and $ANGPT2_{443}$ also suggest that the ANGPT family may be an interesting target for medical intervention.

In the present study we used immunohistochemistry to investigate the localization of ANGPT2 protein. Studies investigating ANGPT2 localization in various tumors in humans have documented the expression in either tumor cells, endothelial cells, or both, depending on the tumor type [25-27]. In the human fetal adrenal gland, ANGPT2 expression was shown to be present in the cortical cells of the definitive zone [14]. In the present study, ANGPT2 protein expression was found in AT cells, but not in normal adrenocortical cells of the zona fasciculata and the zona reticularis, once more suggesting a role for ANGPT2 in AT development. The strong endothelial staining in the vasculature of several canine ATs is likely to represent destabilized blood vessels. Notably, this endothelial staining was present mostly in carcinomas, possibly indicating a role in carcinogenesis.

In order to exhibit an effect, ANGPT2 has to bind to its receptor Tie2. Immunohistochemical localization and overexpression of Tie2 has been consistently shown in tumor vascular endothelial cells, but in some tumor types a positive staining is also present in the tumor cells, the significance of which is yet unknown [28]. Because the results of the present study suggest that ANGPT2 plays a role in AT

development, the presence of the Tie2 receptor in AT had to be investigated. We used immunohistochemistry for this purpose. We detected a strong positive staining for Tie2 in the endothelial lining of the tumor vasculature. This vascular staining pattern might indicate the presence of a paracrine loop in which the ANGPT2 produced by tumor cells and/or vascular endothelial cells binds to Tie2 receptors in the endothelial lining, thus stimulating angiogenesis. In some ATs regions of Tie2-positive tumor cells were detected, possibly indicating the presence of an autocrine ANGPT2-Tie2 loop in these neoplastic cells.

The function of ANGPT2 not only depends on the presence of the Tie2 receptor, but also on the presence of VEGF [29]. In this study, expression analysis revealed no differences in expression of *VEGF* and only a limited decrease in expression of *VEGFR1* levels in adenomas. Nevertheless, in both human [9,30] and canine ATs abundant expression of *VEGF* appears to be present, albeit not different between ATs and normal adrenocortical tissue, which is necessary for an angiogenesis-stimulating effect of ANGPT2.

The expression of *ANGPT2* is known to be regulated by a variety of factors, including hypoxia, several growth factors, VEGF, bFGF and ACTH-cAMP signaling [31-33]. As *VEGF* and *bFGF* did not differ significantly between ATs and normal adrenocortical tissue, and a high level of hypoxia would induce not only *ANGPT2* expression but also *VEGF* expression, our attention was drawn towards the ACTH-cAMP pathway. In the human fetal adrenal, ACTH stimulation targets *ANGPT* expression, preferentially up-regulating *ANGPT2* [14]. Signaling occurs by binding of ACTH to its cell surface receptor, which employs cAMP as its primary intracellular messenger. One of the effects of increased intracellular concentrations of cAMP in adrenocortical cells is increased expression of steroidogenic enzymes via protein kinase A (PKA)-Steroidogenic Factor 1 (SF-1) signaling [34]. Moreover, recently we found that a relatively large proportion of canine ATs carries an activating mutation in GNAS, the gene responsible for cAMP production upon ACTH-stimulation [16]. Based on these considerations, we hypothesized that cAMP may be driving the increased ANGPT2 expression in adrenocortical tumor cells. To test whether raised cAMP concentrations would indeed increase ANGPT2 expression, cell culture experiments were set up. The ideal model would of course consist of a canine, cortisol-producing adrenocortical tumor cell line. Unfortunately, no such cell line is available. The H295R cell line provides the closest *in vitro* model system for AT cells, producing both cortisol and progesterone, and is a stable, well characterized cell line [35]. Stimulation experiments using 8-bromo-cAMP in the H295R cells resulted in a dose-dependent increase of cortisol in the medium, and a time-dependent increase of

ANGPT2 expression, indicating the involvement of cAMP signaling in *ANGPT2* regulation in adrenocortical tumor cells.

Because the human H295R adrenocortical carcinoma cells abundantly express glucocorticoid receptors and progesterone receptors (PR) [30,36], an increase in *ANGPT2* expression due to an increase in cAMP-induced cortisol and/or progesterone secretion had to be investigated. An indirect effect on *ANGPT2* expression by elevated cortisol levels could be excluded by showing that administration of the potent glucocorticoid dexamethasone did not result in increased ANGPT2 expression. Stimulation of H295R cells with progesterone, however, resulted in a transient increase in *ANGPT2* mRNA. In malignant mammary tumors, well known for their high PR expression, a functional relationship appears to exist between angiogenesis and the presence and signaling of PRs. PR-positive mammary tumors have a significantly higher microvessel density [37] and *VEGF* expression in cultured mammary tumor cells is up-regulated by ligand binding induced PR-signaling [38]. The progesterone-induced higher expression of *ANGPT2* in H295R cells may indicate that also in ATs a mechanism may be present in which PR signaling results in a shift of the angiogenic balance towards a pro-angiogenic state.

In conclusion, the increased relative expression of both *ANGPT2-FL* and $ANGPT2_{443}$, the increased *ANGPT2*-to-*ANGTPT1* ratio, the higher expression of $ANGPT2_{443}$ in carcinomas, the protein expression of ANGPT2 in AT cells and in vascular endothelial cells, and the strong Tie2 expression in vascular endothelial cells of carcinomas strongly suggest a role for the ANGPT family in AT development. Induction of *ANGPT2* expression by 8-bromo-cAMP and progesterone provides some insight in the regulation of increased *ANGPT2* expression in ATs. Further research is needed to determine exactly which role ANGPT2 plays in AT development, and whether targeting of the ANGPT2-Tie2 pathway by selective antagonists such as ANGPT2 traps or monoclonal antibodies [39,40] may hold promise as an adjunctive therapeutic option in ATs.

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Chapter 7

Canonical Wnt signaling in canine cortisol-secreting adrenocortical tumors

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Abstract

Activation of the Wnt signaling pathway has been reported in a subset of human ATs, mainly due to mutations in the gene encoding ß-catenin (CTNNB1). The aim of the present study was to retrospectively evaluate the presence of Wnt pathway activation in canine cortisol-secreting ATs. Immunohistochemical ß-catenin staining was evaluated as a marker for canonical Wnt pathway activation in 36 canine cortisol-secreting ATs (11 adenomas, 25 carcinomas). To determine the cause of activation, mutation analysis of CTNNB1 and AXIN2 was performed and the relative mRNA expression of Wnt ligands, Wnt suppressors and Wnt target genes was compared between the ATs and normal adrenals ($n = 15$). Nucleo-cytoplasmic β-catenin staining was detected in 13 of the 36 ATs. Mutation analysis revealed the presence of activating mutations of CTNNB1 in two carcinomas with nucleo-cytoplasmic β-catenin staining. Relative mRNA expression of Wnt-related genes showed a decreased expression of DKK3 ($P = 0.013$), WNT4 ($P < 0.001$) and WNT5B ($P < 0.001$) in carcinomas, and of CCND1 ($P = 0.013$) in adenomas compared to normal adrenals, but no differences in relative expression between ATs with and without nuclear β-catenin staining. In conclusion, nucleo-cytoplasmic β-catenin accumulation was detected in one-third of the canine ATs, suggesting a role for Wnt activation in the tumorigenesis of canine cortisol-secreting ATs.

Introduction

In dogs, hypercortisolism is one of the most frequently observed endocrine disorders, with an estimated incidence of about 1-2 cases per 1000 dogs per year [1]. Of these cases, approximately 15% is due to a cortisol-secreting adrenocortical tumor (AT), which can be either an adenoma or a carcinoma. These tumors are characterized by ACTH-independent hypersecretion of cortisol and uncontrolled growth, the molecular mechanisms of which remain largely unknown. The preferred treatment for canine ATs is complete adrenalectomy, provided no visible metastases or ingrowth in blood vessels are present [2,3]. However, when adrenalectomy is not possible or in case of recurrence, new targeted treatments are needed. For the development of new drugs it is essential to know which cellular pathways play a key role in adrenocortical tumorigenesis.

The canonical Wnt-signaling pathway (Fig. 1) [4] plays an important role in cell survival and proliferation, and aberrantly activated Wnt-signaling is thought to be an important event in the pathogenesis of a variety of tumor types. Accordingly, activation of the Wnt pathway is a frequent observation in human ATs. Somatic activating mutations in the gene encoding β-catenin (*CTNNB1*) are found in about 36% of adenomas and 16% of carcinomas [5,6]. Furthermore, the adrenocortical hyperplasia and tumorigenesis in transgenic mice with constitutive β-catenin activation, indicate a causal role for Wnt activation in AT pathogenesis [7]. Mutations of *CTNNB1* cause stabilization and accumulation of β-catenin, allowing it to translocate to the nucleus and activate the transcription of Wnt target genes [8]. Immunohistochemical evidence of nuclear and/or cytoplasmic β-catenin accumulation is present in approximately 40% of human adrenal adenomas and in 85% of carcinomas [9]. The presence of β-catenin accumulation in ATs without an activating β-catenin mutation suggests the involvement of additional mechanisms for Wnt-activation, such as an increased expression of Wnt ligands, a decreased expression of Wnt inhibitors, or the presence of mutations in components of the β-catenin destruction complex, for instance in *AXIN2* [10,11]. Activation of the cyclic AMP (cAMP) – protein kinase A (PKA) signaling pathway, a well-known phenomenon in the majority of human cortisol-secreting ATs [12,13], can also activate the Wnt pathway [13].

The presence of CTNNB1 mutations and/or immunohistochemical β-catenin accumulation have been shown to be negative prognostic factors on overall and disease-free survival in humans with adrenocortical carcinomas [14]. In the human H295R adrenocortical carcinoma cell line, which carries an activating CTNNB1 mutation, shRNA-induced silencing of β-catenin resulted in decreased cell

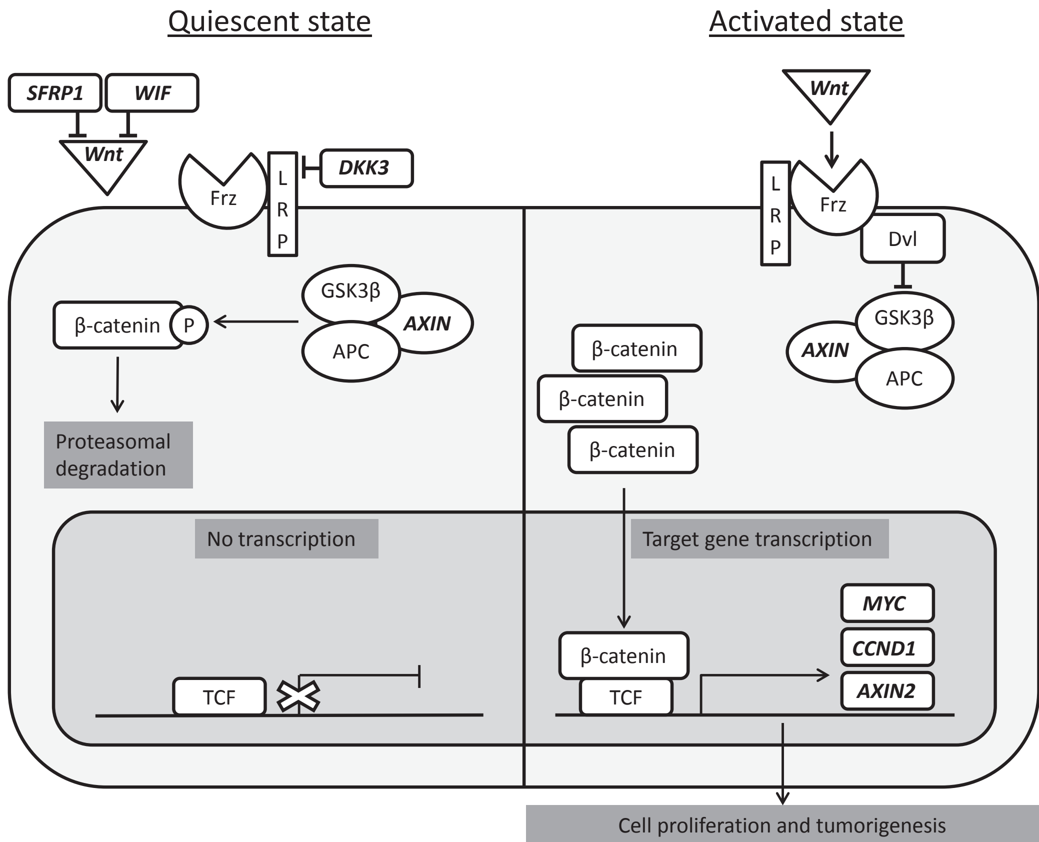

Figure 1

Schematic representation of the canonical Wnt signaling pathway. In a cell with quiescent Wnt signaling (left), secreted frizzled related protein 1 (SFRP1) and Wnt inhibitory factor 1 (WIF) inhibit the binding of Wnt ligands to the frizzled receptor (Frz). Dickkopf 3 (DKK3) inhibits Wnt signaling by its interaction with co-receptor lipoprotein receptor-related protein (LRP). In the cytoplasm, a complex formed by adenomatous polyposis coli (APC), Axin and glycogen synthase 3 β (GSK3β) phosphorylates β-catenin, thus targeting it for proteasomal degradation. Consequently, no target gene transcription takes place.

In a cell with activated Wnt signaling (right), Wnt ligands are able to bind to Frz, upon which disheveled (Dvl) inhibits the actions of the APC-Axin-GSK3β complex. As a result, β-catenin is stabilized and accumulates, allowing it to translocate to the nucleus. There it binds to the T-cell factor (TCF) transcription factor, and induces mRNA expression of target genes such as AXIN2, CyclinD1 (CCND1) and c-MYC (MYC). Through its target genes, activation of the Wnt pathway results in increased cell survival, cell proliferation and tumorigenesis.

Target genes for qPCR analysis in this study are indicated in ***bold italic*** *lettering.*

proliferation and apoptosis *in vitro* and reduced tumor growth *in vivo* in xenograft studies [6], indicating that the Wnt pathway might be promising as a new therapeutic target in adrenocortical cancer.

To evaluate whether the Wnt pathway might also be of prognostic and/or therapeutic relevance in canine ATs, we investigated Wnt pathway activation by

means of immunohistochemical staining for β-catenin. To determine the origin of activation, we evaluated the presence of *CTNNB1* and *AXIN2* mutations, as well as the relative mRNA expression of several Wnt ligands, Wnt inhibitors and Wnt target genes in canine cortisol-secreting adrenocortical adenomas and carcinomas.

Materials and methods

Patient material

Permission to use the AT tissue for this study was obtained from all patient owners and the study was approved by the Ethical Committee of Utrecht University. The patient material used for this study consisted of 36 canine cortisol-producing ATs (11 adenomas and 25 carcinomas). Whole tissue explants of 15 adrenal glands from healthy Beagle dogs, available as archived tissue, were used as controls.

The diagnosis of ACTH-independent hypercortisolism due to an AT in the 36 dogs was based upon (i) elevated urinary corticoid-to-creatinine ratios, which were not suppressible with high doses of dexamethasone, (ii) suppressed or undetectable basal plasma ACTH concentrations [1] and (iii) demonstration of an AT by ultrasonography, computed tomography or both [15]. All ATs were removed by unilateral adrenalectomy. The dogs' age at the time of surgery ranged from 2 to 12 yr (mean, 9 yr). Seven dogs were mongrels and the other dogs were of 22 different breeds. Eighteen of the dogs were male (eight castrated) and 18 female (12 neutered).

After surgical removal of an AT or resection of a normal adrenal gland, the tissue was immediately put on ice and material was inspected and saved for quantitative RT-PCR (qPCR) analysis and histopathology. Tissue fragments for RNA isolation were snap frozen in liquid nitrogen within 10 min and kept at -80°C until further use. The remaining part of the tissue was immersed in formalin for fixation and embedded in paraffin after 24 h to 48 h.

Histopathology

Histopathological evaluation was performed on formalin fixed and paraffin embedded tissue slides for all samples and used to confirm the diagnosis and to classify the tumors. All histological evaluations were performed by a single pathologist. Classification was based on the criteria described by Labelle *et al.* [16]. Classification as a carcinoma was based on histological evidence of vascular invasion,

peripheral fibrosis, capsular invasion, trabecular growth, hemorrhage, necrosis, and single cell necrosis. Typical histological characteristics of adenomas were hematopoiesis, fibrin thrombi, and cytoplasmic vacuolization. Based on these criteria, the tumor group consisted of 11 adenomas and 25 carcinomas.

Follow-up and GNAS mutation status

Of the dogs in the tumor group, follow-up information was available from15 dogs: seven of these dogs developed signs of hypercortisolism within 2.5 yr after surgical removal of the tumor. The recurrence of hypercortisolism was confirmed by endocrine testing, and was caused by metastases in six of these dogs, and by regrowth of the AT in one dog. The remaining eight dogs were in remission for at least 2.5 yr after adrenalectomy.

All ATs had previously been evaluated for the presence of mutations activating the cAMP - PKA pathway [17]. Twelve of the ATs carried an activating mutation of the G-protein stimulatory subunit alpha (*GNAS*), while 24 of the ATs were negative for *GNAS* mutations.

Immunohistochemistry

The presence and localization of β-catenin was evaluated by means of immunohistochemical staining on formalin-fixed, paraffin embedded tissue slides of all ATs and four normal adrenal controls. Tissue slides were rehydrated in a graded series of xylene-alcohol baths. Antigen retrieval was performed using a commercial antigen unmasking solution (H-3300, Vector, Burlingame, CA, USA), which was heated in a microwave for 3 min. To block endogenous peroxidase activity, slides were incubated for 15 min with 3.5% H_2O_2 in PBS. Nonspecific binding sites were blocked using 3% BSA in 0.025% Triton X-100 in TBS for 30 min. A rabbit-anti-human anti β-catenin antibody (AB6302, Abcam, Cambridge, UK) was used, in a 1:400 dilution in 1% BSA in 0.025% Triton X-100 in TBS. Slides were incubated with the primary antibody for 90 min at room temperature. Subsequently, all slides were incubated with anti-rabbit HRP conjugated secondary antibody (Dako K4003) for 30 min. Antibody detection was performed using Dako K3468 HRP substrate. All slides were incubated with DAB for 5 min and subsequently counterstained with heamatoxylin. The specificity of the reaction was verified by omission of the primary antibody. As a positive control, canine colon tissue slides were used. All slides were analyzed in a descriptive manner, to determine the presence and cellular localization of β-catenin staining. The effects of the canonical Wnt signaling pathway are

mediated through nuclear translocation of β-catenin, therefore a positive nucleo-cytoplasmic β-catenin staining was considered indicative of Wnt pathway activation.

Total RNA extraction and reverse transcription

Total RNA for qPCR and sequence analysis was isolated from frozen samples using the RNeasy mini kit (Qiagen, Hilden, Germany), according to manufacturer's protocols. A DNAse step was performed to avoid genomic DNA contamination. RNA concentrations were measured on a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE). Synthesis of cDNA was performed using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA), according to manufacturer's protocols. For all samples, one cDNA reaction was performed without Reverse Transcriptase (RT-), to check for contamination with genomic DNA.

PCR amplification

All primers for PCR amplification were designed using Perl-primer v1.1.14 according to the parameters in the Bio-Rad iCycler manual, and ordered from Eurogentec (Maastricht, The Netherlands). Forward amplification primers were located in the 5' untranslated region (UTR), while reverse primers were located in the 3'UTR. Overlapping primer pairs were used when a gene could not be amplified in one stretch. For all PCR primers a temperature gradient was performed, to determine the optimal annealing temperature. Formation of the proper PCR products was evaluated by gel electrophoresis, to check for the correct product length. In case of correct product lengths, a sequencing reaction was performed to confirm the identity of the transcript, using an ABI3130XL Genetic analyzer according to manufacturer's protocol. After optimization of the protocol, the complete cDNA of the target genes was amplified in all ATs. PCR reactions were performed using Phusion® Hot Start Flex DNA Polymerase (New England BioLabs Inc., Ipswich, MA, USA) on a C1000 Touch thermal cycler (BioRad). All PCR primers and their characteristics are listed in Table 1.

Sequencing

All sequence primers were designed using Perl-primer v1.1.14, and ordered from Eurogentec. All sequence primers and their characteristics are listed in Table 2. Primers were designed in both sense and antisense directions, and located every 300-500 base pairs along the transcript, to obtain complete coverage. All PCR primers were also used as sequence primers. PCR products were amplified for

mRNA PCR primers	Sequence (5’-3’)	Location	Annealing temperature	Product length (bp)
CTNNB1 Fw 94	AGAAAAGCAGCTGTTAGTCAC	94/114	60°C	2401
CTNNB1 Rv 2495	CCTAAACCACTCCCACCCT	2495/2477		
AXIN2 Fw 436	GGGGAAGGGAACAAACAAAC	436/455	59°C	1258
AXIN2 Rv 1694	CTCCTTCTCTTCATCCTCTC	1694/1675		
AXIN2 Fw 1250	GACGGCGAATGTGAGGT	1250/1266	55°C	890
AXIN2 Rv 2140	TTTGAGTAGCAGTAATACTCGG	2140/2119		
AXIN2 Fw 2010	ACGAAGCATGTCCACC	2010/2025	55°C	350
AXIN2 Rv 2360	CTCACTCTCCAGCATCC	2360/2344		
AXIN2 Fw 2211	CTGCCCAAACGAAATGG	2211/2227	55°C	573
AXIN2 Rv 2784	AGTATGTGACGACCAACTC	2784/2766		
AXIN2 Fw 2418	GAGTCGGCCCGTGCGTCCCC	2418/2437	64.5°C	532
AXIN2 Rv 2950	TCGTCCCAGATCTCCTCAAACAC	2950/2928		
AXIN2 Fw 2662	CCACTGGTCAGACAGGA	2662/2678	59°C	361
AXIN2 Rv 3023	GACCCCCGAGGCTCA	3023/3009		

Table 1

PCR primers for the mRNA amplification of canine β-catenin (CTNNB1) and AXIN2. All positions are based on the mRNA sequence, as published on the NCBI website (http://www.ncbi.nlm.nih.gov/). Accession numbers used: CTNNB1: NM_001137652.1, AXIN2: XM_548025.4

Abbreviations: Fw: Forward primer, Rv: Reverse primer

sequencing using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and filtrated using Sephadex G-50 Superfine (Amersham, Buckinghamshire, United Kingdom). Sequencing reactions were performed on an ABI3130XL Genetic analyzer, according to manufacturer’s instructions. The obtained sequences were compared to the consensus mRNA sequence, as denoted on NCBI, using DNAstar Lasergene core suite 9.1 SeqMan software.

Genomic primers	Sequence (5’-3’)	Location	Annealing temperature	Product length (bp)
CTNNB1 F1	GACTTTGTATAGCCTCGTGG	10571442/61	60°C	407
CTNNB1 R1	CAGGTCAGTATCAAACCAGG	10571849/30		
CTNNB1 F2	AGAAAAGCAGCTGTTAGTCAC	10560252/72	60°C	1137
CTNNB1 R2	GAGTTGTAATGGCATAAAATAATA	10561389/66		
AXIN2 F1	GGGCGATCAAGATGGTG	14561124/40	57°C	236
AXIN2 R1	TGCTGCTTCTTGATGCC	14561360/44		

Table 2

PCR primers for the genomic amplification of canine β-catenin (CTNNB1) and AXIN2. All positions are based on the genomic sequence, as published on the NCBI website (http://www.ncbi.nlm.nih.gov/). Accession numbers used: CTNNB1: NC_006605.3, AXIN2: NC_006591.3

Abbreviation: Fw: Forward primer, Rv: Reverse primer

All non-synonymous mutations were confirmed by repeated RNA extraction and sequencing in both sense and antisense directions. For confirmed non-synonymous mutations, whenever possible, the tumor-association was determined by sequencing genomic DNA isolated from peripheral blood using a QIAamp DNA Blood mini kit (51104, Qiagen). Peripheral blood from the patient carrying the mutation was used if available, otherwise genomic DNA from several dogs of the same breed was used. Primers to amplify the affected region in genomic DNA were developed using Perl-primer, and ordered from Eurogentec (Table 3).

Sequence primers	Sequence (5'-3')	Location
CTNNB1 Fw 327	GGCTGCTATGTTCCCTGAGA	327/346
CTNNB1 Fw 688	GGGACCTTGCACAATCTTTCTC	688/709
CTNNB1 Fw 1128	AATGCAGGCTTTAGGGCTTCA	1128/1148
CTNNB1 Fw 1429	CCTGCCATCTGTGCTCTTCGTC	1429/1450
CTNNB1 Fw 1794	TCACAACCGAATCGTAATCAGA	1794/1815
CTNNB1 Fw 2036	ACAAGCCACAGGATTACAAGAA	2036/2057
CTNNB1 Rv 419	GCCAAACGCTGGACATTAG	419/401
CTNNB1 Rv 541	CCACCTGGTCCTCATCATTTA	541/521
CTNNB1 Rv 1548	GGATGGTGGGTGCAGGAGTTTA	1548/1527
CTNNB1 Rv 1944	CTCCGCTTCAATGGCTTCTGC	1944/1924
AXIN2 Fw 816	GCCTGCAACGGATTCAG	816/832
AXIN2 Fw 1663	TGCAGCAGATCCGAGAG	1663/1679
AXIN2 Fw 2062	AGGAGGAGATCGAGGCAGAG	2062/2081
AXIN2 Rv 854	GGTATCCTGCAGGTTCATCTG	854/834
AXIN2 Rv 1306	CTCTTGAAGGACCTGTATCC	1306/1287
AXIN2 Rv 2552	GGGCGGGGTCAGGGGAGGCAT	2552/2532

Table 3

Sequence primers for the mutation analysis of canine β-catenin (CTNNB1) and AXIN2. All positions are based on the mRNA sequence, as published on the NCBI website (http://www.ncbi.nlm.nih.gov/). Accession numbers used: CTNNB1: NM_001137652.1, AXIN2: XM_548025.4

Fw: Forward primer, Rv: Reverse primer

The affected exon was amplified and sequenced as described above. A mutation was considered to be tumor associated if it could be detected only in tumor cDNA, but not in genomic DNA of the patient or the control dogs. For some of the mutations, tumor association could not be determined due to the absence of genomic DNA samples from either patients or control dogs of the same breed. To predict the functional impact, *in-silico* analysis was performed for all observed non-synonymous mutations. The SIFT score predicts tolerability of a substitution based on mathematical operations [18]. On a scale of 0 to 1, scores > 0.05 are predicted to be tolerated and

qPCR primers	Sequence (5’-3’)	Location	Annealing temperature	Product Length (bp)
AXIN2 Fw	GGACAAATGCGTGGATACCT	782/801	60°C	141
AXIN2 Rv	TGCTTGGAGACAATGCTGTT	922/903		
MYC Fw	GCCGGCGCCCAGCGAGGATA	549/568	61°C	108
MYC Rv	GCGACTGCGACGTAGGAGGGCGAGC	656/632		
CCND1 Fw	GCCTCGAAGATGAAGGAGAC	490/509	60°C	117
CCND1 Rv	CAGTTTGTTCACCAGGAGCA	606/587		
WNT2 Fw	GACAGGGATCACAGCCTCTT	295/314	63°C	103
WNT2 Rv	TGGTGATGGCAAACACAACT	397/378		
WNT3 Fw	ATGAACAAGCACAACAACGAG	725/745	61.5°C	138
WNT3 Rv	TTGAGGAAGTCGCCGATAG	873/855		
WNT4 Fw	CGAGGAGTGCCAGTACCAGT	2511/2530	61°C	124
WNT4 Rv	AGAGATGGCGTACACGAAGG	2634/2615		
WNT5A Fw	TGCCACTTGTATCAGGACCA	254/273	61°C	150
WNT5A Rv	GCTGCCTATCTGCATGACC	403/385		
WNT5B Fw	CCCTGTACAGAGACCCGAGA	163/182	61.5°C	98
WNT5B Rv	ACAACTGGCACAGCTTCCTC	260/241		
WNT6 Fw	AGAAGTGGTGGCGGAGCTA	1110/1128	61.5°C	100
WNT6 Rv	CTTGCTGTGGCTGGAACAGT	1209/1190		
WNT7A Fw	GCCTCGACGAGTGTCAGTTT	401/420	60°C	128
WNT7A Rv	GATGATGGCGTAGGTGAAGG	528/509		
WNT10B Fw	TTCTCTCGGGATTTCTTGGA	981/1000	60°C	116
WNT10B Rv	CATTTCCGCTTCAGGTTTTC	1096/1077		
WIF1 Fw	CCGAAATGGAGGCTTTTGTA	799/818	61.6°C	135
WIF1 Rv	ATGCAGAACCCAGGAGTGAC	933/914		
SFRP1 Fw	AGCGAGTTTGCATTGAGGAT	541/560	60°C	106
SFRP1 Rv	TCTTGATGGGTCCCAACTTC	646/627		
DKK3 Fw	GGGCCAGGATTGTAAGTGAA	2172/2191	58°C	114
DKK3 Rv	CATCCAGTCCAGTGCTCTCA	2311/2392		

Table 4

Primer pairs for qPCR amplification of Wnt target genes (CyclinD1 (CCND1), V-Myc Avian Myelocytomatosis Viral Oncogene Homolog (MYC) and AXIN2), Wnt ligands (WNT2, WNT3, WNT4, WNT5A, WNT5B, WNT6, WNT7A, WNT10B) and Wnt inhibitors (Wnt inhibitory factor 1 (WIF1), secreted frizzled related protein 1 (SFRP1) and Dickkopf 3 (DKK3)). All positions are based on the mRNA sequence, as published on the NCBI website (http://www.ncbi.nlm.nih.gov/). Accession numbers used: AXIN2: XM_548025.4, MYC: NM_001003246.2, CCND1: NM_001005757.1, WNT2: XM_849870.2, WNT3: XM_845071.3, WNT4: XM_005617834.1, WNT5A: XM_005632414.1, WNT5B: XM_543883.4, WNT6: XM_005640667.1, WNT7A: XM_844117.3, WNT10B: XM_543687.3, WIF1: XM_538269.4, SFRP1: XM_003639564.2, DKK3: XM_534060.4
Abbreviations: Fw: Forward primer, Rv: Reverse primer

scores < 0.05 are predicted to affect protein function. The PolyPhen2 score predicts functional impact based on Bayesian methods [19]. On a scale of 0 to 1, 0 is predicted to be benign and 1 is predicted to be deleterious. The ConSurf conservation score was used to assess evolutionary conservation of the amino acid residue across species [20]. On a scale from 1 to 9, 1 denotes a highly variable residue and 9 denotes a highly conserved residue.

Quantitative RT-PCR

Primers for quantitative RT-PCR (qPCR) were designed using Perl-primer v1.1.14 according to the parameters in the Bio-Rad iCycler manual, and ordered from Eurogentec. For each primer pair a qPCR temperature gradient was performed, to determine the optimal annealing temperature. Formation of the proper PCR product was confirmed by a sequencing reaction, using the ABI3130XL Genetic analyzer (AB applied Biosystems, Carlsbad, CA) according to manufacturer's protocol. A cDNA pool was made out of all samples, from which a four-fold dilution series was made for use as a standard. The rest of the cDNA was diluted four times with milliQ water, to achieve a working stock. On all of the samples, the relative mRNA expression levels were measured for three transcriptional target genes of the Wnt pathway, nine Wnt ligands and three Wnt inhibitors (Table 4). To correct for differences in the cDNA concentration, ribosomal protein S5 (*RPS5*), *RPS19,* β-glucoronidase (*GUSB*), signal recognition particle receptor (*SRPR*) and hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) were used as reference genes [21,22]. Analysis of the relative expression levels of the reference genes revealed no significant differences between groups and their expression was shown to be stable using GeNorm software [22,23], justifying their use as reference genes.

All qPCR reactions were performed on a MyIQ single color real-time PCR detection system (BioRad). Detection was performed using SYBRgreen supermix (BioRad) and data were analyzed using iQ5 software (BioRad). The raw data were used to calculate the reaction efficiency. Reaction efficiencies between 90% and 110% were accepted. Calculation of normalized relative expression levels for each of the target genes was performed using the ΔΔ-Ct method [24].

Statistical analyses

Statistical analyses were performed using SPSS20 (IBM, Armonk, NY). Because of the non-normal distribution of most of the variables, the non-parametric Mann-Whitney U test was used to compare mRNA expression levels between groups. The relative mRNA expression levels were compared between ATs (adenomas and carcinomas) and normal adrenals, between ATs with and without nuclear β-catenin staining, and

between ATs with and without an activating *GNAS* mutation. In addition, we investigated whether the presence of nuclear β-catenin staining, a GNAS mutation, or CTNNB1 and/or AXIN2 mutations were related to clinical outcome using a Fischer's exact test. For all statistical tests, a *P*-value less than 0.05 was considered significant.

Results

Immunohistochemical evaluation

Immunohistochemical staining for β-catenin in normal adrenal glands (Fig. 2A) showed a predominantly membranous staining pattern in both the zona glomerulosa (ZG) and zona fasciculata (ZF). In some areas of the normal adrenals, a weak granular staining was detected in the cytoplasm, but nucleo-cytoplasmic staining was not present in any of the normal adrenals. Of the 36 ATs, a positive nucleo-cytoplasmic staining for β-catenin was present in 13 (Fig. 2B). In an additional 10 ATs, only a diffuse cytoplasmic granular staining for β-catenin was present, in the absence of nuclear staining (Fig. 2C). The remaining 13 ATs showed only membranous staining, without cytoplasmic or nuclear staining (Fig. 2D). The presence of β-catenin accumulation was not related to the clinical outcome.

Mutation analysis of CTNNB1 and AXIN2

Mutation analysis of the full coding region of *CTNNB1* identified the presence of three different synonymous point mutations in codon 117 (GCT > GCC, in 2 ATs), codon 221 (CTT > CTG, in 2 ATs) and codon 576 (GCC > GCT, in 5 ATs). Synonymous point mutations could be present in either homozygous or heterozygous form. Four different non-synonymous mutations of *CTNNB1* were detected (Table 5). Heterozygous missense point mutations were detected in codons 717 (R717H) and 698 (L698I). Neither of these mutations was associated with β-catenin accumulation, SIFT and Polyphen2 scores predicted both mutations to be benign and tolerated, and ConSurf scores indicated low and average residue conservation, respectively. A heterozygous nonsense point mutation in the highly conserved codon 30 (Y30*), resulting in a premature stop codon, was detected in one carcinoma with nuclear β-catenin staining. This mutation was confirmed to be tumor associated by sequencing genomic DNA of the affected dog. Finally, a heterozygous 26-bp frameshift deletion, resulting in a downstream premature stop codon (Y30Wfs*40) was detected in one carcinoma with nuclear β-catenin staining.

Mutation analysis of the full coding region of *AXIN2* identified the presence of two different synonymous mutations, which were present in codon 658 (GGC > GGT, heterozygous in one AT) and codon 816 (GCG > GCA, either heterozygous or homozygous, present in 10 ATs). Three different non-synonymous point mutations were detected in codons 103 (K103R), 610 (G610R) and 754 (A754V), each present in two ATs (Table 5). Based on the ConSurf scores, the affected residues were highly variable across species, and all three mutations were predicted to be benign and tolerated based on SIFT and Polyphen2 scores.

There were no ATs carrying mutations in both CTNNB1 and AXIN2.

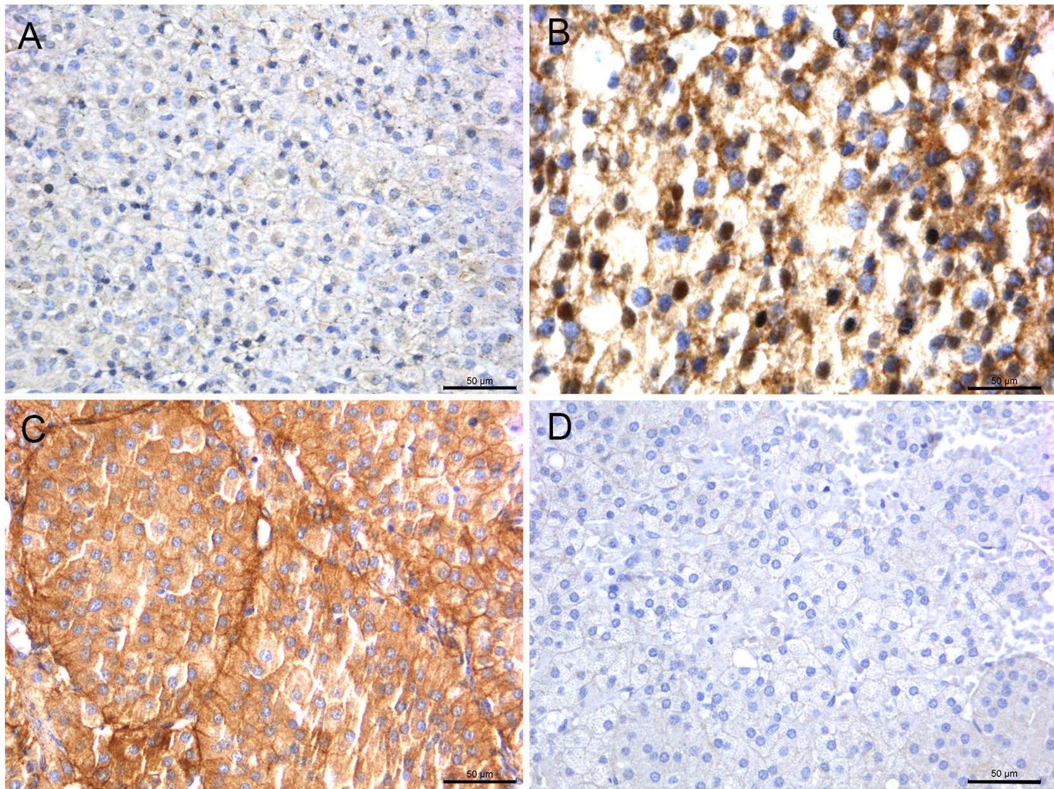

Figure 2

Immunohistochemical staining for β-catenin. Representative examples of immunohistochemical staining for β-catenin, only membranous staining was detected in the zona fasciculata of normal canine adrenal cortex (A). Ten of the ATs exhibited only membranous staining (B), 13 ATs showed only cytoplasmic accumulation without nuclear staining (C), and 10 ATs showed a nucleo-cytoplasmic staining pattern (D). Bars represent 50 μm

Relative expression of Wnt-target genes, Wnt ligands and Wnt inhibitors

Analysis of the relative mRNA expression levels of the Wnt-target genes in adenomas and carcinomas, when compared to normal adrenals (Fig. 4A), revealed a significantly decreased expression of *CCND1* in the adenoma group (0.4-fold, P = 0.013). No significant differences were detected in the relative expression levels of *AXIN2* and *MYC*. For the Wnt ligands, a significant decrease was detected in the relative expression levels of WNT4 (0.2-fold, P < 0.001) and WNT5B (0.3-fold, P < 0.0001) in carcinomas. For the Wnt inhibitors, a significant decrease of DKK3 expression was detected in carcinomas (0.5-fold, P = 0.013). In adenomas, no

Mutation	Number of ATs	Tumor association	β-catenin staining	SIFT score	Polyphen2 score	ConSurf score
<u>*CTNNB1*</u>						
R717H	1 (C)	-	-	Tolerated 0.36	Benign 0.176	5
L698I	1 (A)	NA	-	Tolerated 0.28	Benign 0.000	1
Y30*	1 (C)	+	+	NA	NA	8 F
Y30Wfs*40	1 (C)	NA	+	NA	NA	8 F
<u>*AXIN2*</u>						
K103R	2 (C)	-	1 + 1 -	Tolerated 0.52	Benign 0.020	Variable 2
G610R	2 (1A, 1C)	NA	+	Tolerated 0.11	Benign 0.001	Variable 1
A754V	2 (1A, 1C)	NA	1 + 1 -	Tolerated 0.24	Benign 0.104	Variable 1

Table 5

Overview of the non-synonymous mutations detected in CCND1 and AXIN2. For each mutation, the table lists the number of positive adrenocortical tumors (ATs), tumor-association (based on genomic DNA sequencing of either the affected patient or multiple healthy dogs from the same breed), the presence or absence of nucleo-cytoplasmic immunohistochemical β-catenin staining, SIFT scores (on a scale of 0 to 1, scores > 0.05 are predicted to be tolerated and scores < 0.05 are predicted to affect protein function), Polyphen2 scores (on a scale of 0 to 1, 1 = predicted to be benign and 1 = predicted to be deleterious) and ConSurf conservation scores (on a scale from 1 to 9, 1 = highly variable and 9 = highly conserved) of the affected residues.
Abbreviations: CTNNB1: β-catenin, A: adenoma, C: carcinoma, NA: non-applicable, F: predicted functional residue

significant differences in the relative expression of any of the Wnt ligands or inhibitors were detected. When comparing the ATs with and without nucleo-cytoplasmic β-catenin accumulation, no significant differences were detected in the relative expression levels of any of the investigated genes, neither were there any significant differences in gene expression when comparing ATs with nucleo-cytoplasmic or cytoplasmic β-catenin staining with negative ATs.

Relation to GNAS mutation status

In the group of ATs carrying an activating *GNAS* mutation (Fig. 2B), a significant decrease of *CCND1* expression (2.45-fold, *P* = 0.002) was present when compared to the group without *GNAS* mutation. For the Wnt ligands, ATs carrying an activating *GNAS* mutation displayed a significantly decreased relative expression of WNT2 (11.0-fold, P = 0.032), WNT3 (6.9-fold, P < 0.001), WNT5B (3.1-fold, P = 0.006) and WNT7A (3.1-fold, P = 0.035), while the relative expressions of WNT5A (2.8-fold, P = 0.009) and WNT10B (6.3-fold, P = 0.013) were significantly increased in the GNAS-mutant group when compared to the group without GNAS mutation. For the Wnt inhibitors, SFRP1 expression was significantly decreased in ATs carrying an activating GNAS mutation (5.2-fold, P = 0.038), whereas no significant differences were found in expression levels of the other genes encoding Wnt inhibitors. There was also no relation between nuclear β-catenin accumulation and the presence of activating GNAS mutations. None of the ATs with a mutation in CTNNB1 or AXIN2 carried an activating GNAS mutations, suggesting these mutations to be mutually exclusive.

Discussion

The present study elucidates the role of canonical Wnt signaling in canine cortisol-secreting ATs for the first time. We detected the presence of nucleo-cytoplasmic β-catenin accumulation in 13 of the 36 ATs (36%). Additionally, two of the 13 ATs with a positive nucleo-cytoplasmic β-catenin staining (15%) carried non-synonymous CTNNB1 mutations, expected to affect protein function. Therefore, like in humans, Wnt pathway activation is present in a subset of canine ATs.

In addition to nuclear β-catenin accumulation, we investigated activation of the Wnt pathway by determining the mRNA expression of AXIN2, MYC and CCND1, known transcriptional targets of β-catenin [25-28]. Surprisingly, no increase in relative expression of the target genes was detected in ATs and neither was the expression increased in the group of ATs with positive nuclear β-catenin staining. As nuclear β-catenin staining is considered to be the distinguishing mark of canonical Wnt pathway activation, our results indicate that in canine ATs AXIN2, CCND1 and MYC may not be the optimal target genes for the detection of Wnt pathway activation.

Supporting this notion, in human ATs no increase of these genes has been detected in studies reporting unbiased gene expression arrays [29,30] and MYC expression is

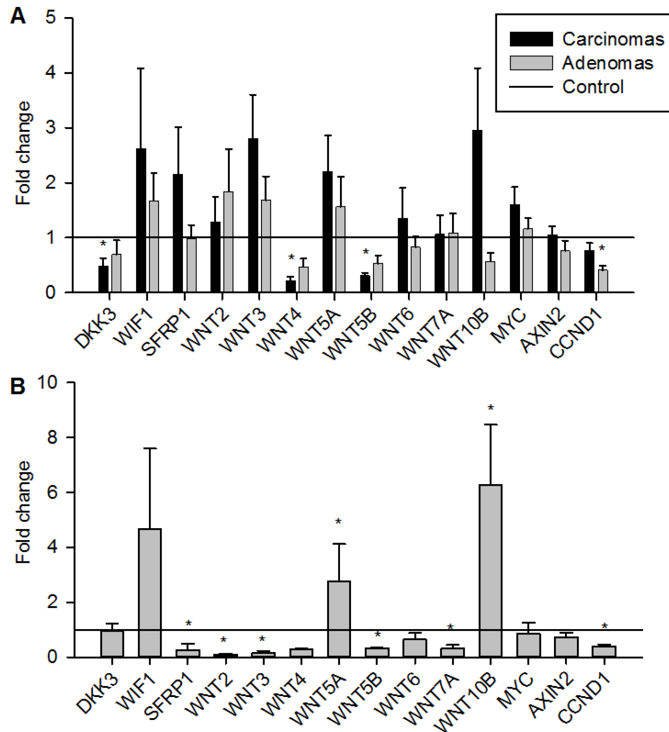

Figure 4

Relative mRNA expression of target genes in 11 canine cortisol-secreting adenomas and 25 carcinomas. Relative expression in adenomas and carcinomas, as a fold change toward the mean expression in 15 normal adrenal glands, as measured by qPCR and calculated using the 2^-ΔΔCt method (A). Relative expression levels in ATs with an activating G-protein stimulatory subunit alpha (GNAS) mutation, as a fold change toward the mean expression in wild type ATs (B). Bars represent mean fold change +/- SEM. Significant changes (P < 0.05) are marked with an asterisk.

decreased in human adrenocortical carcinomas [29,31]. Different studies have compared target gene expression levels between human ATs with and without Wnt pathway activation (as indicated by activating *CTNNB1* mutations and/or nuclear β-catenin staining). One study did not reveal increased *AXIN2* or *MYC* expression in ATs with an activated Wnt pathway [32], while other studies did find increased *AXIN2* mRNA levels in human ACCs with Wnt pathway activation [33,34]. Interestingly, in these studies the majority of ACCs with Wnt pathway activation also carried activating *CTNNB1* mutations, which was not the case in our canine cohort. Also, the increased *AXIN2* expression was not detected in human adrenocortical adenomas with Wnt activation [33]. Theoretically, these differences might explain the discrepancy between the results in human studies and our canine cohort. Microarray

or transcriptome studies on a large and well-characterized group of canine ATs would be of great value to provide evidence of which target genes are the best indicators of Wnt pathway activation in these tumors.

The only Wnt target gene for which altered mRNA expression levels were detected, was *CCND1*, the relative expression of which was decreased in adenomas. Repression of *CCND1* expression is thought to be a hallmark of cell differentiation [27], which may explain its low expression in adrenal adenomas. Based on histological characteristics and tumor behavior adrenocortical adenomas represent a highly differentiated tumor type. In line with this, a decrease in *CCND1* expression was detected in human adrenocortical adenomas when compared to carcinomas [35].

To determine potential causes of Wnt pathway activation in canine ATs, all tumors were screened for the presence of activating mutations in *CTNNB1*. We detected the presence of four different non-synonymous mutations, all of which were present in single ATs. Only the tumor-associated Y30* mutation has previously been reported in a human adrenocortical carcinoma in which the Wnt pathway was activated [36]. In this tumor, transcription from an alternative in-frame ATG downstream of the stop codon presumably caused the formation of a truncated protein, which was detected using western blotting and lacked the amino acids encoded by exons 1-3. The truncated protein has an intact transactivation domain, but lacks the phosphorylation domain which targets β-catenin for ubiquitination and degradation, leading to protein stabilization and constitutive activation. The Y30Wfs*40 mutation we detected also results in a premature stop codon, and might thus be activating the pathway in the same way. This is supported by the presence of nuclear accumulation of β-catenin in the affected AT. In contrast, the R717H and L698I substitutions are unlikely to represent functional changes, as these mutations occurred in less conserved residues, were predicted to be benign and tolerated based on *in-silico* analysis and were not associated with nucleo-cytoplasmic β-catenin staining.

Mutation analysis of *AXIN2* revealed the presence of three different non-synonymous point mutations, each present in 2 ATs, none of which has previously been reported. Despite the presence of nucleo-cytoplasmic β-catenin staining in three of these ATs, the variability of the affected residues and the *in-silico* analysis of the mutations do not support a functional significance for the detected mutations in *AXIN2*. One of the limitations of a cDNA approach for mutation analysis is the inability to detect truncating mutations leading to mRNA degradation. Therefore, a DNA based sequencing approach would be needed to fully exclude the possibility of functional *AXIN2* mutations in canine ATs.

The presence of nuclear β-catenin accumulation in ATs that do not carry *CTNNB1* mutations, calls for an alternative mechanism of Wnt-activation, such as the differential expression of Wnt ligands or inhibitors or other ligand independent routes of activation. Expression analysis revealed no significant changes in adenomas, while in carcinomas a decrease in *DKK3*, *WNT4* and *WNT5B* expression was detected, comparable to the expression profile in human childhood adrenocortical carcinoma [31]. While a decrease of Wnt inhibitor *DKK3* expression could contribute to Wnt activation, the decreased expression of Wnt ligands *WNT4* and *WNT5B*, the lack of increased expression of the other Wnt ligands and/or decreased expression of the other Wnt inhibitors, as well as the lack of differential expression between ATs with and without nuclear β-catenin, indicate that the activation of the Wnt pathway in canine ATs without *CTNNB1* or *AXIN2* mutations cannot be explained by differential expression of these Wnt ligands or inhibitors.

To determine whether activation of the cAMP-PKA pathway is associated with Wnt activation as it is in human ATs [13,36,37], Wnt-related genes were compared between ATs with and without activating mutations of *GNAS*. The decreased expression of most Wnt ligands and of *CCND1* is not in accordance with an increase of Wnt-signaling upon PKA activation in canine ATs. This difference between humans and dogs may be due to the fact that PKA activation in human ATs is mainly due to mutations of PKA regulatory subunit 1A (*PRKAR1A*) while in dogs only *GNAS* mutations are detected [13,17]. Mutations of *PRKAR1A* and *GNAS* were recently reported to lead to different pathway alterations, and human ATs carrying a *GNAS* mutation had lower Wnt ligand and *CCND1* expression than *PRKAR1A*-mutant ATs [38]. Thus, although the *GNAS* mutations clearly affect mRNA expression levels of Wnt-related genes in canine ATs, the expression profile of these genes and the lack of association between *GNAS* mutations and nuclear β-catenin staining do not indicate *GNAS*-mutations as a factor in Wnt pathway activation in canine ATs.

In conclusion, our results indicate stabilization of β-catenin in a subset of canine cortisol-secreting ATs, which may play a role in tumorigenesis. Our results do not indicate increased Wnt ligand expression or PKA-signaling as important factors in Wnt-activation of canine ATs. Activating *CTNNB1* mutations were detected only in 2 ATs. Future research is needed to elucidate the cause of Wnt-activation in the ATs that do not carry a mutation of *CTNNB1*, and to show whether recently described compounds that can inhibit Wnt signaling in preclinical models [39] may be beneficial as a therapeutic strategy in canine ATs.

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Chapter 8

Insulin-like growth factor – phosphatidylinositol 3 kinase signaling in canine cortisol-secreting adrenocortical tumors

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Abstract

Background: Hypercortisolism is a common endocrine disorder in dogs, caused by a cortisol-secreting adrenocortical tumor (AT) in approximately 15% of the cases. In human adrenocortical carcinomas (ACC) the activation of the phosphatidylinositol 3 kinase (PI3K) signaling pathway through insulin-like growth factor (IGF) signaling represents a promising therapeutic target.

Objectives: To investigate the involvement of PI3K signaling in the pathogenesis of canine ATs and to identify pathway components that may hold promise as future therapeutic targets or as prognostic markers.

Animals: Analyses were performed on 36 canine cortisol-secreting ATs (11 adenomas and 25 carcinomas) and 15 normal canine adrenal glands.

Methods: mRNA expression analysis was performed for PI3K target genes, PI3K inhibitor phosphatase and tensin homolog (*PTEN*), IGFs, IGF receptors, IGF binding proteins and epidermal growth factor (EGF) receptors. Mutation analysis was performed on the genes encoding *PTEN* and PI3K catalytic subunit (*PIK3CA*).

Results: Target gene expression indicated PI3K activation in carcinomas, but not in adenomas. No amino acid changing mutations were detected in *PTEN* or *PIK3CA* and no significant alterations in IGF-II or IGFR1 expression were detected. In carcinomas *ERBB2* expression tended to be higher than in normal adrenals, and a higher expression of inhibitor of differentiation 1 and 2 (*ID1* and *ID2*) was detected in carcinomas with recurrence within 2.5 years after adrenalectomy.

Conclusions and clinical importance: Based on these results, ERBB2 might be a promising therapeutic target in canine ATs, while ID1 and 2 might prove valuable as prognostic markers and as therapeutic targets.

Introduction

Hypercortisolism is one of the most common endocrine disorders in dogs [1]. Approximately 15% of the spontaneous cases of canine hypercortisolism is due to a cortisol-secreting adrenocortical adenoma or carcinoma [1]. Therapeutic options for canine adrenocortical tumors (ATs) are limited: complete adrenalectomy of the affected adrenal gland is the treatment of choice, provided that no metastases are present at the time of presentation [2,3]. However, surgery is not possible or successful in all cases, and tumor recurrence and metastasis occur regularly [4]. Options for medical management are limited to mitotane, a chemotherapeutic agent, or trilostane, which can only alleviate the symptoms of hypercortisolism. The lack of reliable prognostic markers further complicates treatment.

A pathway with the potential to provide both therapeutic targets and prognostic markers is the phosphatidylinositol-3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) signaling pathway (Fig. 1). The PI3K pathway is one of the most frequently activated signal transduction pathways in human cancer, the activation of which has also been documented in human ATs [5-7]. Pathway activation is initiated through receptor tyrosine kinases, such as the type-1 insulin-like growth factor (IGF) receptor (IGFR1) or dimers of the epidermal growth factor (EGF) receptor family (EGFR, ERBB2-4), and counteracted by the competitive PI3K inhibitor phosphatase and tensin homolog (PTEN). Upon activation of the PI3K pathway, phosphorylated AKT (p-AKT) and its downstream effectors stimulate cell proliferation, survival and growth through transcriptional and posttranslational mechanisms [5].

The PI3K pathway contains multiple targets for therapeutic intervention, the choice of which depends on the mode of activation. In human adrenocortical carcinomas (ACC), the frequent overexpression of *IGF-II* and *IGFR1* indicate IGF-signaling as a likely mode for PI3K activation [8-10]. Selective IGFR1 kinase inhibitors could thus be of benefit, and indeed have shown anti-tumor effects both in cell culture studies and pre-clinical and early phase clinical trials of human ACC [6,10]. For EGFR-induced PI3K pathway activation, several specific inhibitors have already been approved for clinical use in humans [11]. Activation of the PI3K pathway may also occur downstream of the receptors, for instance due to mutations in the genes encoding PTEN or the PI3K catalytic subunit (PIK3CA) or due to decreased expression of PTEN [12,13]. In these cases, single or dual inhibitors of PIK3CA and mTOR could be employed [14]. In a human ACC cell line, the use of these compounds has resulted in a decrease of cell proliferation and cortisol secretion [15,16].

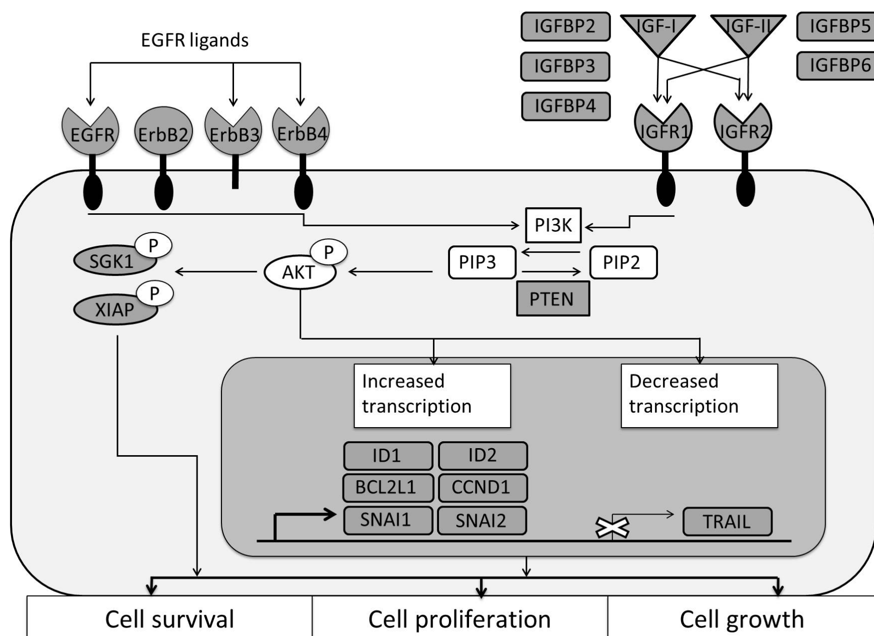


Figure 1

Schematic representation of the PI3K signaling pathway. Upon ligand binding the epidermal growth factor receptors (EGFR and ERBB 2-4) form homo- and heterodimers, activating their tyrosine kinase domain. The IGF receptors (IGFR1 and IGFR2) are activated by the binding of insulin-like growth factor (IGF) I or II. IGF actions are modulated by high affinity IGF binding proteins (IGFBP 2-6). IGFR1 and the EGF receptors relay their intracellular signal to phosphatidylinositol 3 kinase (PI3K), which converts the inactive PIP2 to the active PIP3. Phosphatase and tensin homolog (PTEN) counters PI3K action by converting PIP3 back to PIP2. PIP3 production leads to the phosphorylation of AKT to phospho-AKT (P-AKT), which in turn activates SGK1 and XIAP through phosphorylation, while it induces mRNA expression of target genes ID1, ID2, SNAI1, SNAI2, BCL2L1 and CCND1, and inhibits mRNA expression of TRAIL. Through its target genes, activation of the PI3K pathway results in increased cell survival, proliferation and growth. Target genes for qPCR analysis in this study are indicated in dark grey.

Abbreviations: SGK1: serum glucocorticoid regulated kinase 1, XIAP: X-linked inhibitor of apoptosis, ID1: inhibitor of differentiation 1, ID2: inhibitor of differentiation 2, SNAI1: snail, SNAI2: slug, BCL2L1: B-cell lymphoma 2 related protein, CCND1: cyclin D1, TRAIL: tumor necrosis factor superfamily member 10

In human ACC, *IGF-II* is the most frequently and strongly overexpressed gene [17,18], while in adenomas overexpression occurs only rarely [18,19]. Additionally, high *IGF-II* expression in human ACC is associated with aggressive tumor behavior and increased risk of metastasis [20,21]. Therefore, in humans IGF-II is a diagnostic and prognostic marker for ATs.

The aim of the present study was to investigate the involvement of the PI3K signaling pathway in the pathogenesis of canine cortisol-secreting ATs, in order to identify pathway components that may hold promise as future therapeutic targets or may

serve as prognostic markers. Pathway activation was evaluated by means of target gene expression analysis, while mRNA expression analysis and mutation analysis were used to indicate the mode of activation.

Materials and Methods

Patient material

Patient material used in this study consisted of 36 canine cortisol-secreting ATs and 15 whole tissue explants of normal canine adrenal glands. All normal adrenal glands from healthy dogs were available as archived tissue for comparison with AT tissue obtained from patients. The tumor group consisted of histologically confirmed ATs from patients with clinical signs of hypercortisolism, referred to the Department of Clinical Sciences of Companion Animals of the Faculty of Veterinary Medicine in Utrecht between 2001 and 2012. The diagnosis of ACTH-independent hypercortisolism due to a cortisol-secreting AT was based upon (i) elevated urinary corticoid-to-creatinine ratios, which were not suppressible with high doses of dexamethasone, (ii) suppressed or undetectable basal plasma ACTH concentrations [1] and (iii) demonstration of an AT by ultrasonography, computed tomography or both [22]. All dogs subsequently underwent unilateral adrenalectomy. The dogs' age at the time of surgery ranged from 2 to 12 yr (mean, 9 yr). Seven dogs were mongrels and the other dogs were of 22 different breeds. Eighteen of the dogs were male (8 castrated) and 18 female (12 neutered).

After resection, all ATs and normal adrenal glands were immediately put on ice for inspection, and material was saved for quantitative RT-PCR (qPCR) analysis and histopathology. Fragments for RNA isolation were snap frozen in liquid nitrogen within 10 min after resection and stored at -80°C until further use. The remaining part of the tissue was immersed in formalin for fixation and embedded in paraffin after 24 h to 48 h. Permission to use the AT tissue for this study was obtained from all patient owners and the study was approved by the Ethical Committee of Utrecht University.

Histopathology

Histopathological evaluation was performed on formalin fixed and paraffin embedded tissue slides of all samples and used to confirm the diagnosis and to classify the tumors. All histological evaluations were performed by a single pathologist. Classification was performed based on the criteria described by Labelle

et al. [23]. Classification as a carcinoma was based on histological evidence of vascular invasion, peripheral fibrosis, capsular invasion, trabecular growth, hemorrhage, necrosis, and single cell necrosis. Typical histological characteristics of adenomas were hematopoiesis, fibrin thrombi, and cytoplasmic vacuolization. Based on these criteria, the tumor group consisted of 11 adenomas and 25 carcinomas.

Follow-up

Of the dogs in the tumor group, follow-up information was available from 15 dogs with histologically confirmed carcinomas: 7 of these dogs developed signs of hypercortisolism within 2.5 yr after surgical removal of the tumor. The recurrence of hypercortisolism was confirmed by endocrine testing, and was caused by metastases in 6 of these dogs, and by regrowth of the AT in 1 dog. The remaining 8 dogs were in remission for at least 2.5 yr after adrenalectomy.

Total RNA extraction and reverse transcription

Total RNA for quantitative RT-PCR analysis was isolated from the adrenal tissue using the RNeasy mini kit (Quiagen, Hilden, Germany), according to manufacturer's protocols. An additional DNAse step was performed to avoid genomic DNA contamination. RNA concentrations were measured on the NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE). Synthesis of cDNA was performed using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA), according to manufacturer's protocols. For all samples, one cDNA reaction was performed without reverse transcriptase (RT-), to check for contamination with genomic DNA.

Quantitative RT-PCR

Primers for qPCR were designed to detect the mRNA expression levels of PI3K pathway target genes (Table 1), PI3K pathway inhibitor PTEN and major components of the IGF and EGF axis (Table 2). Primer design was performed using Perl-primer v1.1.14 according to the parameters in the Bio-Rad iCycler manual, and primers were ordered from Eurogentec (Maastricht, the Netherlands). For all primer pairs a temperature gradient was performed, to determine the optimal annealing temperature. Formation of the proper PCR products was confirmed by a sequencing reaction, using the ABI3130XL Genetic analyzer (AB applied biosystems, Carlsbad, CA) according to manufacturer's protocol.

For mRNA expression analysis of the EGF receptors, PI3K target genes and PTEN, a 10× diluted pool of cDNA samples was used to create a 4-fold standard dilution series. The remaining cDNA was diluted 5 times with milliQ water, to achieve a

working stock. Reactions were performed on a CFX384 real-time PCR detection system (Bio-Rad). The following genes were measured: EGF receptor (*EGFR*), erythroblastic leukemia viral oncogene homolog 2-4 (*ERBB2-4*), snail (*SNAI1*), slug (*SNAI2*), B-cell lymphoma 2 related protein (*BCL2L1*), cyclinD1 (*CCND1*), inhibitor of differentiation 1 and 2 (*ID1* and *ID2*), tumor necrosis factor superfamily member 10 (*TNFSF10* or *TRAIL*), serum glucocorticoid regulated kinase 1 (*SGK1*), X-linked inhibitor of apoptosis (*XIAP*) and *PTEN*. To correct for differences in cDNA concentration, ribosomal protein S5 (*RPS5*), *RPS19*, small proline rich protein (*SPRP*) and hypoxantine phosphoribosiltransferase (*HPRT*) were used as reference genes [24,25].

For mRNA expression analysis of *IGF-I*, *IGF-II*, *IGFR1*, *IGFR2*, IGF binding proteins (*IGFBP 2-6*) and the insulin receptor (*INSR*), an undiluted pool of cDNA samples was used to create a 4-fold standard dilution series. The rest of the cDNA was diluted 10 times with milliQ water, to achieve a working stock. Reactions were performed on a MyIQ single color real-time PCR detection system (Bio-Rad). To correct for differences in cDNA concentration, *RPS5*, *RPS19*, *SPRP* and beta-glucuronidase (*GUSB*) were used as reference genes [24,25]. Detection was performed using SYBRgreen supermix (Bio-Rad) and data were analyzed using CFX Manager 3.0 (Bio-Rad) for the CFX384 real-time PCR data and using iQ5 software (Bio-Rad) for the MyIQ single color real-time PCR data. The raw data were used to calculate the reaction efficiency. Reaction efficiencies between 90% and 110% were accepted. Analysis of the relative expression levels of the reference genes revealed no significant differences between groups and reference gene expression was shown to be stable using GeNorm software [21], justifying the use as reference genes. Calculation of normalized relative expression levels for each of the target genes was performed using the $2^{-\Delta\Delta CT}$ method [26].

Mutation analysis

Mutation analysis was performed on *PTEN* and *PIK3CA*. Primers for PCR amplification (Table 3) and sequence primers (Table 4) were designed using Perl-primer v1.1.14 according to BioRad iCycler parameters, and ordered from Eurogentec. Sequence primers were located along the entire transcript, at a distance of 300-500 bp apart, or closer together when needed for complete coverage. Amplification primers were located in the 3' and 5'untranslated regions (UTRs) of the gene to ensure amplification of the complete coding region. For *PTEN*, the canine 3' UTR is not annotated and was deduced from the human 3' UTR and the canine genomic *s*equence. If a gene could not be amplified in one stretch, overlapping primer pairs were used. All amplification primers were tested on a pool of adrenal

qPCR primers		Sequence (5’-3’)	Annealing temperature	Product length (bp)
SNAI1	*Fw*	CAA GAT GCA CAT CCG AAG C	61.6 °C	133
	Rv	CAG TGG GAG CAG GAA AAC		
SNAI2	*Fw*	CTT CAC TCC GAC TCC AAA CG	60 °C	148
	Rv	TGG ATT TTG TGC TCT TGC AG		
BCL2L1	*Fw*	GGG GTG GTG AGG TAC AAA AA	61.6 °C	112
	Rv	CTG GGT CTA GCG TCC AAA AG		
ID1	*Fw*	CTC AAC GGC GAG ATC AG	59.5 °C	135
	Rv	GAG CAC GGG TTC TTC TC		
ID2	*Fw*	GCT GAA TAA ATG GTG TTC GTG	60.5 °C	114
	Rv	GTT GTT CTC CTT GTG AAA TGG		
CCND1	*Fw*	GCC TCG AAG ATG AAG GAG AC	60 °C	117
	Rv	CAG TTT GTT CAC CAG GAG CA		
TNFSF10	*Fw*	GCT GAT CCT CAT CTT CAC TG	62 °C	90
	Rv	TCC TGC ATC TGC TTC AG		
SGK11	*Fw*	TGG GCC TGA ACG ACT TTA TT	62 °C	124
	Rv	GAG GGG TTG GCA TTC ATA AG		
XIAP	*Fw*	ACT ATG TAT CAC TTG AGG CTC TGG TTT C	54 °C	80
	Rv	AGT CTG GCT TGA TTC ATC TTG TGT TAT G		

Table 1

Quantitative RT-PCR primer pairs for the detection of PI3K target genes: Snail (SNAI1), Slug (SNAI2), B-cell lymphoma 2 related protein (BCL2L1), inhibitor of DNA binding 1 and 2 (ID1 and ID2), CyclinD1 (CCND1), serum/glucocorticoid regulated kinase 1 (SGK11), tumor necrosis factor superfamily member 10 (TNFSF10 or TRAIL) and X-linked inhibitor of apoptosis (XIAP). All positions are based on the mRNA sequence, as published on the NCBI website. Accession numbers used: SNAI1: XM_543048.1, SNAI2: XM_543048.1, CDH1: XM_536807.3, BCL2L1: NM_001003072.1, ID1: XM_847117.2, ID2: XR_134413.1, CCND1: NM_001005757.1, SGK11: XM_003432525.1, TNFSF10: NM_001130836, XIAP: XM_003435664.1

Abbreviations: Fw: Forward primer, Rv: Reverse primer

samples, to determine the optimal annealing temperature. Correct product formation was evaluated by means of gel electrophoresis and sequencing.

After PCR optimization, target genes in all samples were amplified on a C1000 Touch thermal cycler[c] using Phusion® Hot Start Flex DNA Polymerase (New England BioLabs Inc., Ipswich, MA, USA). The PCR products were amplified for sequencing using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and filtrated using Sephadex G-50 Superfine (Amersham, Buckinghamshire, United Kingdom). Sequencing reactions were performed on an ABI3130XL Genetic analyzer, according to manufacturer’s instructions. The sequences obtained were aligned to the NCBI consensus mRNA sequence using DNAstar Lasergene core suite 9.1 SeqMan software. Mutations altering the amino-

qPCR primers		Sequence (5’-3’)	Annealing temperature	Product length (bp)
EGFR	*Fw*	CTG GAG CAT TCG GCA	53 °C	107
	Rv	TGG CTT TGG GAG ACG		
ERBB2	*Fw*	CGT GCT GGA CAA TGG AGA CC	64 °C	126
	Rv	CCG CTG AAT CAA GAC CCC TC		
ERBB3	*Fw*	TAG TGG TGA AGG ACA ACG GCA G	64 °C	103
	Rv	GGT CTT GGT CAA TGT CTG GCA G		
ERBB4	*Fw*	CAG TTC TTG TGT GCG TGC CTG	70 °C	121
	Rv	ATG ATC CTG TGC CGA TGC C		
PTEN	*Fw*	AGA TGT TAG TGA CAA TGA ACC T	62 °C	101
	Rv	GTG ATT TGT GTG TGC TGA TC		
IGF-I	*Fw*	TGT CCT CCT CGC ATC TCT T	58 °C	125
	Rv	GTC TCC GCA CAC GAA CTG		
IGF-II	*Fw*	CTT CTG GAG ACC TAC TGT GC	61 °C	128
	Rv	CTG CTT CCA GGT GTC GTA TTG		
IGFR1	*Fw*	CAT GCC TTG GTC TCC CTG T	60 °C	129
	Rv	GGT GGT CCC AAT CCC AAA G		
IGFR2	*Fw*	GAG TTC AGC CACG AGA C	54°C	94
	Rv	GCA TTG TCA CCA TCA AGG		
IGFBP2	*Fw*	GAT CTC CAC CAT GCA CCT TC	60 °C	127
	Rv	GCT GCC CGT TCA GAG ACA TCT TG		
IGFBP3	*Fw*	CTG CAC ACG AAG ATG GAT GT	61 °C	127
	Rv	TAT TCC GTC TCC CGC TTG TA		
IGFBP4	*Fw*	AGC CTG CAG CCC TCT GAC A	59 °C	120
	Rv	TGG TGC TGC GGT CTC GAA T		
IGFBP5	*Fw*	TCG CAG AAA GAA GCT GAC C	60 °C	131
	Rv	GAA GCC TCC ATG TGT CTG C		
IGFBP6	*Fw*	CAA TCC TGG TGG TGT CC	54 °C	136
	Rv	AGA AGC CCT TAT GGT CAC		
INSR	*Fw*	GTG ACA GAC TAT TTA GAT GTC CC	60 °C	166
	Rv	ACT CAG GGT TTG AAG AAG C		

Table 2

Quantitative RT-PCR primer pairs for the detection of genes encoding components of the IGF- and EGF axis, and PTEN: Epidermal growth factor receptor (EGFR), erythroblastic leukemia viral oncogene homolog 2-4 (ERBB2-4), phosphatase and tensin homolog (PTEN), insulin-like growth factor 1 and 2 (IGF-I and IGF-II), IFG receptor type 1and 2 (IGFR1 and IGFR2), IGF binding protein 2-6 (IGFBP 2-6) and insulin receptor (INSR). All positions are based on the mRNA sequence, as published on the NCBI website. Accession numbers used: EGFR: XM_533073.3, ERBB2: NM_001003217.1, ERBB3: XM_538226.4, ERBB4: XM_003640190.2, PTEN: NM_001003192.1, IGF-I: XM_848024.1, IGF-II: XM_858107.1, IGFR1: XM_853622.1, IGF-IIR: NM_001122602, IGFBP2: XM_545637.2, IGFBP3: XM_548740.2, IGFBP4: XM_845091.1, IGFBP5: XM_847792.1, IGFBP6: XM_844250, INSR: XM_542108.

Abbreviations: Fw: Forward primer, Rv: Reverse primer

acid sequence were confirmed by repeat RNA extraction and sequencing in both sense and antisense directions.

Statistical analyses

Statistical analyses were performed using SPSS20 (IBM, Armonk, NY). Because of the non-normal distribution of most of the variables, the non-parametric Mann-Whitney U test was used to compare mRNA expression levels between groups. The relative mRNA expression levels were compared between ATs (adenomas and carcinomas) and normal adrenals and between ATs with and without recurrent disease. For the first comparison, a Bonferroni correction was applied and $P < 0.025$ was considered significant, while for the latter comparison, a P value < 0.05 was considered significant.

Results

Relative mRNA expression of PI3K target genes

To evaluate whether activation of the PI3K pathway was present in canine cortisol-secreting ATs, mRNA expression analysis was performed on a selection of 10 known target genes of the pathway. Based on the literature, PI3K activation would increase

PCR primers		Sequence (5'-3')	Annealing temperature	Product length
cf_PTEN	*Fw75*	TCC TCC TTC CTC TCC AG	55°C	748
	Rv822	TGA ACT TGT CTT CCC GTC		
	Fw718	CAA TGT TCA GTG GCG GA	55°C	743
	Rv1460	CGA GAT TGG TCA GGA AGA G		
cf_PIK3CA	*F1*	TTT CTG CTT TGG GAC AGC	55°C	1536
	R1537	CTG GGA ACT TTA CCA CAC TG		
	Fw1438	TGC TGA ACC CTA TTG GTG	55°C	449
	Rv1887	TAC AGT CCA GAA GCT CCA		
	Fw1516	GCA GTG TGG TAA AGT TCC	55°C	1843
	Rv3359	CAG TCT TTG CCT GTT GAC		

Table 3
PCR primers pairs for amplification of phosphatase and tensin homolog (PTEN) and phosphatidylinositol 3 kinase, catalytic subunit alpha (PIK3CA). All positions are based on the mRNA sequence, as published on the NCBI website. Accession numbers used: PTEN: NM_001003192.1, PIK3CA: XM_545208.3

mRNA expression of *ID1* [27,28], *ID2* [29], *SNAI1* and *SNAI2* [30], *CCND1* and *BCL2L1* [31]. In contrast, lower mRNA expression of *TRAIL* [31,32] would be expected (Fig. 1). The activation of SGK1 and XIAP occurs by means of phosphorylation, so for these genes no effect on mRNA expression would be expected [33,34] (Fig. 1). In canine adrenocortical carcinomas, the relative mRNA expression levels when compared to those in normal adrenals (Fig. 2A) were significantly higher for *ID1* (2.1-fold, P = 0.021) and *SNAI1* (1.8-fold, P = 0.024), and significantly lower for *SGK1* (0.5-fold, P = 0.009). The relative mRNA expression of *TRAIL* (0.6-fold, P = 0.028) tended to be lower. Thus, in carcinomas three of the seven transcriptionally regulated target genes showed a change in accordance with pathway activation, while the mRNA expression of the other genes remained unchanged.

Sequence primers		Sequence (5'-3')
cf_PTEN	*Fw474*	CAC TGT AAA GCT GGA AAG GG
	Fw1157	TGT AGA GGA GCC ATC AAA CC
	Rv249	CAA AGG GTT CAT TCT CTG GG
	Rv595	TGT CTC TGG TCC TTA CTT CC
	Rv1285	CCT GTA TAC GCC TTC AAG TC
cf_PIK3CA	*Fw375*	GTA ATT GAG CCA GTA GGC
	Fw707	CAA CCA TGA CTG TGT TCC
	Fw1153	TCT ATC ATG GAG GAG AAC CC
	Fw1483	CTC CAT GCT TAG AGT TGG AG
	Fw1767	CTA CCC AAA CTG CTT CTG
	Fw2183	TAG GCA AGT TGA GGC TAT GG
	Fw2582	TGG TTG TCT GTC AAT CGG
	Fw2806	TGG GAA TTG GAG ATC GTC
	Fw2963	GAT TAG TAA AGG AGC CCA GG
	Rv358	AAA GCC GAA GGT CAC AAA GC
	Rv730	GTT CTG GAA CAC AGT CAT GG
	Rv1236	TAG CCA TTC ATT CCA CCT GG
	Rv1750	CAC AAT AGT GTC TGT GGC TC
	Rv2205	TTC CAT AGC CTC AAC TTG CC
	Rv2642	GTG TGA GAA TTT CGC ACC
	Rv2993	TTT GTG CAT TCC TGG GCT
	Rv3271	CAT GCT GCT TAA TGG TGT GG

Table 4

Sequencing primers for the mutation analysis of phosphatase and tensin homolog (PTEN) and phosphatidylinositol 3 kinase, catalytic subunit alpha (PIK3CA). All positions are based on the mRNA sequence, as published on the NCBI website. Accession numbers used: PTEN: NM_001003192.1, PIK3CA: XM_545208.3

Abbreviations: Fw: Forward primer, Rv: Reverse primer

In adenomas, the mRNA expression of none of the genes showed a significant change consistent with PI3K pathway activation (Fig. 2A). The only significant finding was a lower relative expression of CCND1 (0.42-fold, P = 0.004), when compared to that of normal adrenal glands. When comparing dogs with recurrent disease within 2.5 yr to dogs remaining in remission (Fig. 2B), a significantly higher expression of both ID1

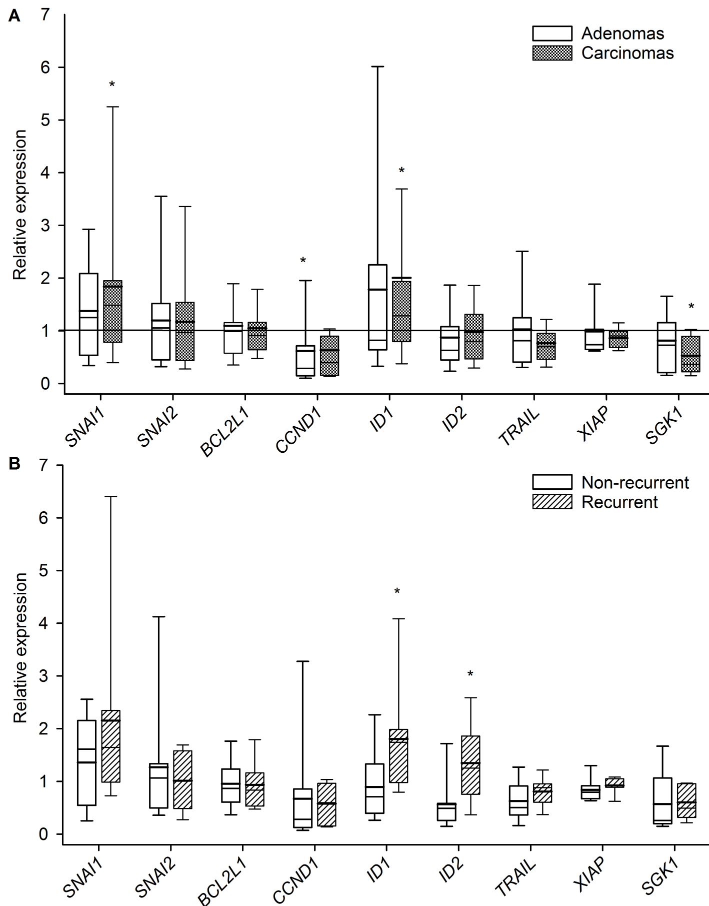


Figure 2

Box-and-whisker plot describing the relative mRNA expression of PI3K target genes in 37 canine ATs and 15 normal adrenal glands, as measured by qPCR and calculated using the $2^{-\Delta\Delta CT}$ method. Relative expression in adenomas and carcinomas when compared to normal adrenal glands (A). Relative expression in ATs with recurrence within 2.5 yr after adrenalectomy, when compared with ATs remaining in remission for at least 2.5 yr (B).

Significant changes (P < 0.05 for A and P < 0.025 for B) are marked with an asterisk. Abbreviations: N: normal adrenal gland, C: adrenocortical carcinoma, A: adrenocortical adenoma, SNAI1: Snail, SNAI2: Slug, BCL2L1: B-cell lymphoma 2 related protein, ID1 and ID2: inhibitor of DNA binding 1 and 2, CCND1: CyclinD1, SGK11: serum/glucocorticoid regulated kinase 1, TNFSF1: tumor necrosis factor superfamily member 10, XIAP: X-linked inhibitor of apoptosis

(2.0-fold, $P = 0.033$) and *ID2* (2.4-fold, $P = 0.019$) was detected in dogs with recurrent disease. No significant changes were detected in the expression of other target genes.

Relative mRNA expression of PTEN and the components of IGF and EGF signaling

To look for a potential cause of PI3K activation, the mRNA expression levels of the components of the IGF axis, the EGF receptors and PI3K-inhibitor *PTEN* were evaluated. Analysis of the components of the IGF axis in carcinomas (Fig. 3A) revealed a significantly higher expression of *IGFBP*2 (5.8-fold, $P = 0.001$) and a significantly lower expression of *IGFBP5* (0.5-fold, $P = 0.001$). Likewise, in adenomas, *IGFBP2* expression was significantly higher (7.2-fold, $P = 0.013$) and *IGFBP5* expression was significantly lower (0.4-fold, $P < 0.001$). No significant differences in the expression levels of *IGF-II* or the *IGFR1* were detected.

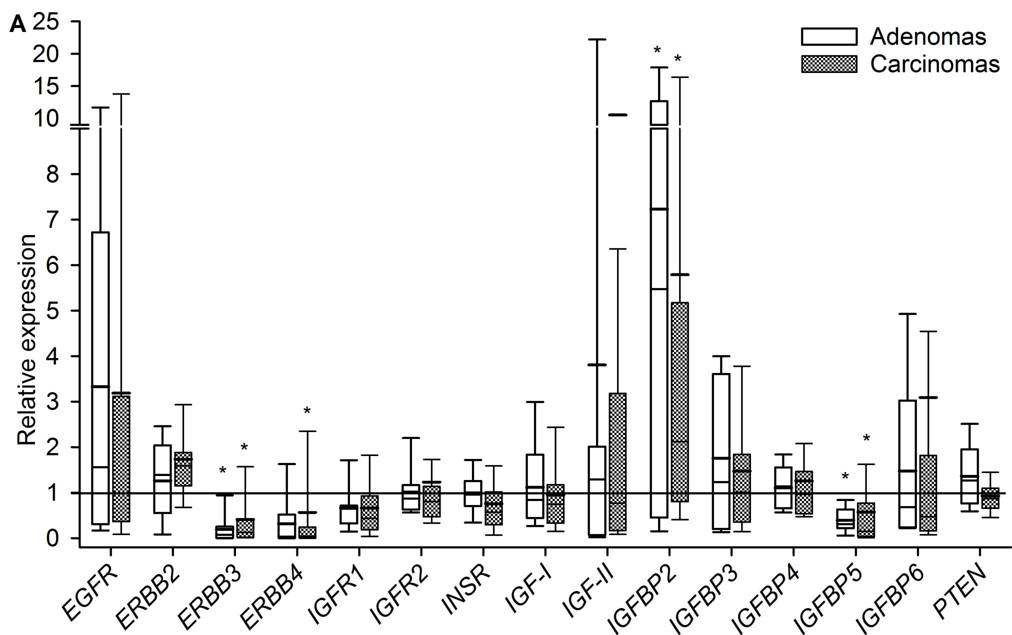
Analysis of the EGF receptors in carcinomas revealed a tendency to a higher *ERBB2* expression (1.7-fold, $P = 0.027$) and a significantly lower expression of *ERBB3* (0.4-fold, $P = 0.003$). In adenomas, the only significant change was a lower *ERBB3* expression (0.2-fold, $P = 0.001$).

When comparing dogs with recurrent disease within 2.5 yr to dogs remaining in remission (Fig. 3B), a significantly higher expression of *IGF-I* (2.7-fold, $P = 0.042$), *IGFBP5* (6.8-fold, $P = 0.042$) and *INSR* (2.3-fold, $P = 0.040$) was detected.

Mutation analysis of PTEN and PIK3CA

To determine whether inactivating mutations of *PTEN* or activating mutations of *PIK3CA* might be responsible for activation of the PI3K pathway, mutation analysis was performed. Mutation analysis of *PTEN* revealed the presence of 1 silent mutation in codon 325 (CTC>CTT), which was present in 6 ATs and occurred in both homo- and heterozygous form. No amino acid changing mutations were detected in of any of the ATs.

Mutation analysis of *PIK3CA* revealed the presence of 3 different heterozygous silent mutations (codon 149 CCA>CCC, codon 438 TCT>TCA and codon 842 GTG>GTT) in 3 different ATs. No amino acid changing mutations were detected in any of the ATs.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Carcinomas
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ERBB2
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IGFBP6
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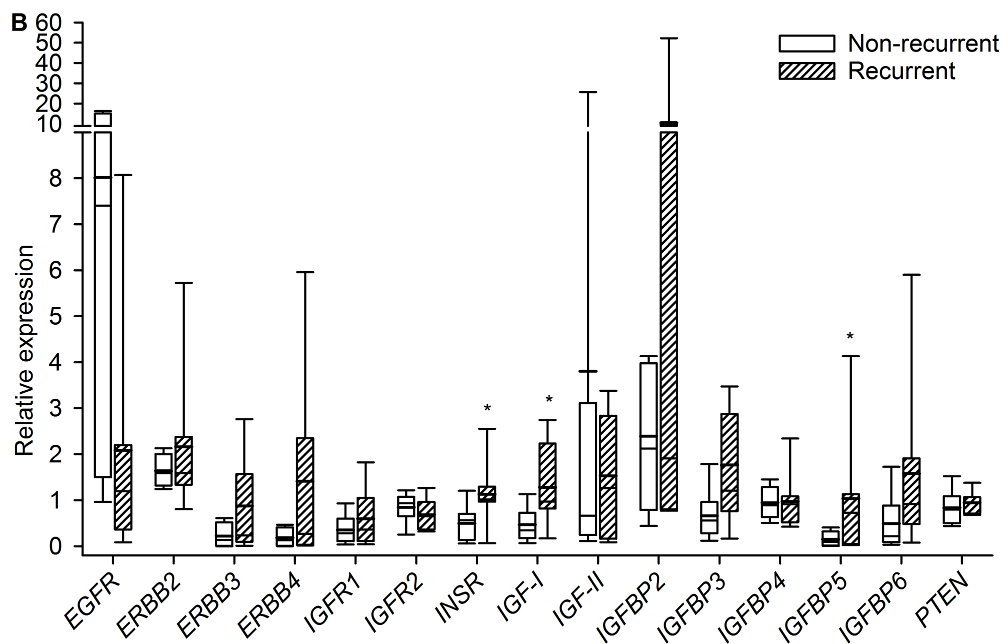
B
Relative expression
Non-recurrent
Recurrent
EGFR
ERBB2
ERBB3
ERBB4
IGFR1
IGFR2
INSR
IGF-I
IGF-II
IGFBP2
IGFBP3
IGFBP4
IGFBP5
IGFBP6
PTEN

Figure 3

Box-and-whisker plot describing the relative mRNA expression of genes encoding components of the IGF- and EGF axis, and PTEN in 37 canine ATs and 15 normal adrenal glands, as measured by qPCR and calculated using the $2^{-\Delta\Delta CT}$ method. Relative expression in adenomas and carcinomas when compared to normal adrenal glands (A). Relative expression in ATs with recurrence within 2.5 yr after adrenalectomy, when compared with ATs remaining in remission for at least 2.5 yr (B).

Significant changes (P < 0.05 for A and P < 0.025 for B) are marked with an asterisk.

Abbreviations: N: normal adrenal gland, C: adrenocortical carcinoma, A: adrenocortical adenoma, PTEN: phosphatase and tensin homolog, IGF-I and 2: insulin like growth factor 1 and 2, IGFR1: IFG receptor type 1, IGF-IIR: IGF receptor type 2, IGFBP2-6: IGF binding protein 1-6, INSR: insulin receptor, EGFR: epidermal growth factor receptor, ERBB2-4: erythroblastic leukemia viral oncogene homolog 2-4

Discussion

In the present study, we aimed to investigate activation of the PI3K pathway in canine cortisol-secreting adrenocortical adenomas and carcinomas, in order to identify both potential therapeutic targets and prognostic markers for use in canine ATs. The presence of PI3K activation was assessed by means of target gene expression analysis. In adenomas, none of the target genes showed a significant change consistent with PI3K pathway activation, and there was even a significantly lower expression of *CCND1*. This may be explained by the fact that adrenocortical adenomas, based on histological characteristics and tumor behavior, are a highly differentiated tumor type, and repression of *CCND1* expression is thought to be a hallmark of cell differentiation [35]. In line with this, lower *CCND1* expression was detected in human adrenocortical adenomas when compared to carcinomas [36]. In contrast, in canine adrenocortical carcinomas all target genes that showed a significant alteration in mRNA expression were altered in accordance with PI3K activation.

The main modes of activation of the PI3K pathway are through intracellular alterations of signaling pathway components or through receptor tyrosine kinase signaling. With regard to intracellular pathway alterations, activating mutations of *PIK3CA* and inactivating mutations of *PTEN* are well-documented [14]. In this study, no amino acid changing mutations were detected in either *PIK3CA* or *PTEN* and no overall decreased expression of *PTEN* was detected in ATs.

With regard to receptor tyrosine kinase induced activation, one of the most strongly documented changes that can activate the PI3K pathway in human ACC, is activation of the IGF axis. Multiple studies have demonstrated that in human ACC, *IGF-II* is the most overexpressed gene [19,37]. In the healthy individual, one of the alleles the *IGF-II* locus is epigenetically silenced postnatally, but in ACC genetic and epigenetic

alterations in the 11p15 locus cause both alleles to be active, resulting in *IGF-II* overexpression [17]. Likewise, a high IGF-II protein expression is characteristic for human ACC [17,18]. The mechanism by which high IGF-II expression can lead to activation of the PI3K pathway is through binding the IGFR1, which like IGF-II frequently shows increased expression in human ACC [8,9]. Taken together, these data suggest that in human ACC changes in the IGF axis could be responsible for PI3K activation. This notion is supported by studies in H295R human adrenocortical carcinoma cells, where IGF-II - IGFR1 signaling results in an increase of p-AKT [6,7,38]. Remarkably, the present study did not reveal a higher expression of either *IGF-II* or *IGFR1* in canine ATs.

For *IGFBP2* a higher mRNA expression was detected in canine adenomas and carcinomas. The IGFBPs function as regulatory components of IGF signaling [39], and some studies have indicated that high IGFBP2 expression may contribute to ACC pathogenesis. In murine Y1 AT cells, long-term increased *IGFBP2* levels enhanced the malignant phenotype [40] and in *IGF-II* overexpressing human ACC an increased expression of IGFBP2 has been reported [20]. However, the significance of high *IGFBP2* expression in the absence of *IGF-II* overexpression is unknown. Theoretically, high IGFBP2 levels could lead to higher IGF-II availability and thus increase IGFR1/PI3K signaling. However, the fact that *IGFBP2* overexpression was also detected in adenomas in the absence of increased target gene expression, does not support a functional role of *IGFBP2* overexpression in PI3K activation in dogs.

In contrast, *IGFBP5* showed an overall lower expression in ATs, but a 6.8-fold higher expression in those carcinomas showing recurrence within 2.5 yr after adrenalectomy. In different human cancer types, *IGFBP5* overexpression has been noted as a prognostic marker. In particular, in breast cancer, high *IGFBP5* expression is associated with a shorter recurrence-free, metastasis-free and overall survival and *IGFBP5* overexpression in breast cancer cell lines conferred resistance to IGFR1 inhibition [41]. Our results therefore indicate the mRNA expression of *IGFBP5* as a relevant prognostic factor in canine adrenocortical carcinomas.

An alternative mechanism for receptor tyrosine kinase-induced PI3K pathway activation is EGFR signaling. In this respect, the tendency toward a higher *ERBB2* mRNA expression in canine adrenocortical carcinomas is interesting. ERBB2 (also known as HER2) is a receptor tyrosine kinase that lacks a ligand binding domain, and functions through heterodimerization with the other EGF receptors. Aberrant ERBB2 activation, for instance due to receptor overexpression, results in activation of the PI3K pathway, growth stimulation and tumorigenesis in different tumor types, of which ERBB2-positive breast cancer is the most prominent example [11,42].

Different drugs targeting ERBB2 have been approved for clinical use in cancer treatment, including antibodies that inhibit heterodimerization (Trastuzumab and Pertuzumab [43,44]) and tyrosine kinase inhibitors that affect both EGFR (i.e. ERBB1) and ERBB2 activity (Lapatinib and Afatinib [45,46]). Recent studies suggest that simultaneously targeting EGFR and ERBB2 further increases antitumor activity [11]. Based on the higher expression of *ERBB2*, this receptor might be a promising new therapeutic target in canine adrenocortical carcinomas, either singly or in combination with EGFR inhibition.

Apart from therapeutic targets, we also aimed to investigate the pathway components for potential new prognostic markers. In this regard, apart from *IGFBP5*, *SGK1* and *ID1* and *ID2* are also worth mentioning. The relative mRNA expression of *SGK1* was significantly lower in carcinomas. At a first glance, this seems surprising, as *SGK1* is activated by PI3K signaling only at the protein level, through phosphorylation [33]. Recently however, *SGK1* microdeletions and low *SGK1* mRNA expression have been reported in human cortisol-secreting ATs, but not in non-secreting or aldosterone-secreting ATs [47-49]. Low SGK1 protein expression was found to be an independent prognostic factor for shorter overall survival [48]. Although the present study did not reveal significant differences in *SGK1* mRNA expression between dogs with and without recurrent disease, the low *SGK1* expression in cortisol-secreting carcinomas does suggest a functional role for *SGK1*. Further studies involving SGK1 protein expression and overall survival analyses are needed to determine whether SGK1 expression might prove to be a prognostic marker in these tumors.

In the group of dogs with recurrent disease, we did find an increase of both *ID1* and *ID2* expression. This is in accordance with studies reporting on an association between high *ID1* and *ID2* expression and poor prognosis in multiple different human tumor types [50]. ID proteins are thought to keep cells in a poorly differentiated, proliferative state, and therefore a role for ID1 and 2 in the pathogenesis of malignant canine ATs appears likely. Based on our results, ID1 and 2 show promise as new prognostic markers for canine ACC. Future studies are needed to determine whether targeting IDs might also be feasible as a new therapeutic option [51,52].

In conclusion, our results suggest the presence of PI3K activation in canine cortisol-secreting adrenocortical carcinomas, but not in adenomas. In contrast to one of the most prominent features of human ACC, no significant alterations in *IGF-II* or *IGFR1* expression were detected. Therefore, our results do not indicate that inhibition of IGF signaling is likely to prove a successful strategy for canine ATs. However, we did

find a higher expression of *ERBB2*, providing a pre-clinical rationale for studying the potential of ERBB2 inhibition, as used in ERBB2 positive breast cancer, in canine adrenocortical carcinomas. Finally, the lower expression of *SGK1* in carcinomas and the higher expression of *IGFBP5*, *ID1* and *ID2* in ATs with early recurrence may represent an important step in the search for prognostic markers for canine cortisol-secreting adrenocortical carcinomas.

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Chapter 9

General discussion

Cortisol-secreting tumors of the adrenal cortex occur both in humans and in dogs, and are characterized by their uncontrolled growth and excessive cortisol secretion, which because of a lack of explanation have thus far been characterized as autonomous [1]. Treatment options in dogs are limited [2-4], and no reliable prognostic markers have been identified. The 1000-fold higher incidence of cortisol-secreting ATs in dogs when compared to humans, raises the question whether the dog might be a good spontaneous animal model for human adrenocortical carcinoma (ACC) [1].

The aims of this thesis were:

a) To investigate molecular pathways, alterations of which may lead to autonomous growth and cortisol secretion in canine cortisol-secreting ATs
b) To search for potential new therapeutic targets and prognostic markers in canine cortisol-secreting ATs
c) To evaluate the value of dogs with a spontaneous cortisol-secreting AT as model for human ACC

In this chapter, we discuss the outcome of the studies investigating the molecular pathways, therapeutic targets and prognostic markers in canine ATs, and their implications for the treatment of these dogs. Additionally, the results are compared with the present knowledge on these topics in human ACC and the implications of the observations in this PhD study for the use of the dog as a spontaneous model organism for human ACC are discussed.

Molecular pathways involved in ACTH-independent hypersecretion of cortisol

One of the main characteristics of cortisol-secreting ATs is the so called autonomous, or ACTH-independent, secretion of cortisol [1]. In the normal adrenal cortex, an ACTH signal from the pituitary is needed to induce cortisol synthesis and secretion. However, in case of a cortisol-secreting AT, the high levels of cortisol inhibit pituitary ACTH secretion by means of a negative feedback mechanism, resulting in low to an undetectable plasma ACTH concentration. Despite the lack of ACTH stimulation, the tumor cells continue to produce excessive amounts of cortisol. In dogs, the mechanisms resulting in ACTH-independent cortisol secretion remained to be elucidated.

A pathway with the potential to explain ACTH-independent cortisol secretion is the cyclic AMP (cAMP) – protein kinase A (PKA) signaling pathway. In the normal adrenal cortex, the G-protein coupled ACTH receptor (melanocortin 2 receptor, MC2R) serves to initiate the intracellular signaling cascade that leads to cortisol

secretion [5]. Binding of ACTH to the MC2R activates the G-protein stimulatory subunit alpha (Gsalpha), which induces adenylate cyclase to produce cAMP. Subsequently, PKA is activated, which serves to activate several nuclear transcription factors. Aberrant activation of this pathway could therefore result in ACTH-independent cortisol secretion. Accordingly, in human cortisol-secreting ATs, alterations in this pathway occur frequently [6]. A common cause of cAMP/PKA activation in human ATs is an inactivating mutation of the gene encoding PKA regulatory subunit 1 A (*PRKAR1A*) [7,8]. Recently, activating mutations of the gene encoding PKA catalytic subunit A (*PRKACA*) have also been demonstrated in a large percentage of human ATs [9-11]. Other, less common mechanisms of cAMP/PKA activation in human ATs include activating mutations in *GNAS*, the gene encoding for Gsalpha [7,12], or inactivating mutations in the genes encoding phosphodiesterases (*PDE*), which inactivate cAMP by converting it to 5'AMP [6,13]. Recently, several studies have underlined the importance of cAMP pathway activation in the pathogenesis of human cortisol-secreting ATs [14].

In the study described in **Chapter 3**, no amino acid changing mutations in *MC2R* and *PRKAR1A* were detected, but approximately one-third of the canine cortisol-secreting ATs exhibited an activating *GNAS* mutation [15]. By inducing constitutive cAMP production, these mutations could well explain the ACTH-independent hypersecretion of cortisol in the affected subset of ATs.

In the subset of ATs negative for *GNAS* mutations, the ACTH-independent cortisol secretion remains unexplained. Therefore, it would be interesting to determine whether other mutations within the cAMP/PKA pathway, for instance in *PRKACA* or *PDE*, might account for these cases. Alternatively, ACTH-independent cortisol-secretion in these cases could be caused by alterations upstream from cAMP, such as altered eutopic expression of hormone receptors coupled to this pathway. Like the MC2R, somatostatin (SST) and dopamine (DA) receptors are G-protein coupled receptors. All SST receptors (SSTR1-5) and the D_2-like DRs (DRD_2, D_3 and D_4) have an inhibitory influence on hormone secretion and cell proliferation through the G-protein inhibitory subunit (Gi), which inhibits adenylate cyclase and thus cAMP production [16,17]. In contrast, D_1-like dopamine receptors (DRD_1 and D_5) stimulate adenylate cyclase through Gsalpha, and thus induce cAMP formation and hormone secretion [17]. In human ATs, overexpression of both SSTRs and DRs has been shown [18-20].

In the study described in **Chapter 4**, overexpression of *SSTR* or *DR* was not detected in canine cortisol-secreting ATs. Canine adrenocortical carcinomas exhibited a lower DRD_2 and *SSTR1* expression and adenomas exhibited lower *SSTR1* expression. This might indicate tumor escape from the inhibiting influence of SST and DA signaling.

Moreover, in cortisol-secreting carcinomas with recurrence within 2.5 yr, a significant 4-fold overexpression of DRD_1 was detected. As DRD_1 is known to activate adenylate cyclase through GSalpha, its overexpression may represent an alternative mechanism for cAMP activation and ACTH-independent cortisol secretion.

A key mediator of ACTH-cAMP induced cortisol synthesis in the adrenal cortex is the orphan nuclear receptor steroidogenic factor 1 (SF-1) [21,22]. SF-1 plays a key role in the development of steroidogenic tissues and in the adult adrenal cortex functions as an obligate activator of the majority of cytochrome P450 steroid hydroxylases and steroidogenic acute regulatory (StAR) protein [21,23]. Consequently, SF-1 null mice die shortly after birth of adrenocortical insufficiency [24,25]. In human cortisol-secreting ACC *SF-1* overexpression has been reported [26,27]. Based on these observations, overexpression of *SF-1* might well be involved in the ACTH-independent hypersecretion of cortisol in ATs. However, in the study described in **Chapter 5**, neither significant differences in *SF-1* expression were detected between cortisol-secreting ATs and normal adrenals, nor a higher mRNA expression of SF-1 was found in dogs with an activating GNAS mutation [28]. These results suggest that differences in mRNA expression of *SF-1* do not contribute to ACTH-independent hypercortisolism in dogs with a cortisol-secreting AT. However, it is not fully clear what molecular mechanism is responsible for the interplay between cAMP signaling and SF-1 in the regulation of cortisol secretion [21]. While a cAMP-induced regulation of expression has been proposed, other studies have contradicted this hypothesis [29-31]. Alternatively, cAMP signaling may recruit SF-1 on a posttranscriptional level. A mechanism in which SF-1 is activated by the binding of phosphatidic acid upon cAMP signaling has been suggested [32-34]. Therefore, phospholipid profiling in primary cultures of cortisol-secreting ATs could prove valuable to investigate the role of cAMP - SF-1 signaling in ACTH-independent hypercortisolism.

Although the link between activated cAMP signaling and SF-1 activity in canine ATs remains to be elucidated, it is clear that activation of the cAMP signaling pathway is a common factor in both human and canine cortisol-secreting ATs. Interestingly, the main mechanisms contributing to cAMP/PKA activation are different in human and canine ATs. This could reflect in other molecular differences between human and canine ATs, as a recent study showed that the downstream pathways affected by cAMP/PKA activation differ depending on the molecular origin of the activation [35].

While the activation of cAMP/PKA signaling clearly is of major importance in the development of ACTH-independent hypercortisolism, other cAMP-independent mechanisms may also be of influence. One such factor that was recently identified in human cortisol-secreting ATs, is serum glucocorticoid regulated kinase 1 (SGK1), one

of the target genes of the phosphatidylinositol 3 kinase (PI3K) pathway [36]. Recent studies identified *SGK1* microdeletions and low *SGK1* mRNA expression in human cortisol-secreting ATs, but not in non-secreting or aldosterone-secreting ATs [36-38]. Additionally, an inverse relationship between SGK1 expression and cortisol secretion was detected [36]. This suggests an involvement of low *SGK1* expression in ACTH-independent cortisol secretion, possibly because low SGK1 levels could reduce the intra-adrenal negative feedback loop, leading to uninhibited cortisol secretion [36]. In the study described in **Chapter 8**, a significantly lower relative expression of *SGK1* was detected in cortisol-secreting carcinomas when compared to normal adrenals, suggesting a similar phenomenon as seen in human ATs. Further studies are needed to elucidate the role of *SGK1* in ACTH-independent cortisol secretion and the molecular mechanisms resulting in lower expression of *SGK1* in ATs of humans and dogs.

Molecular pathways involved in uncontrolled tumor growth and metastasis

Apart from the ACTH-independent cortisol secretion, the other major characteristic of both human and canine cortisol-secreting ATs is their uncontrolled growth [1,39]. In dogs, cortisol-secreting carcinomas are typically larger than 2 cm, while adenomas are generally smaller [40]. Metastases occur regularly, and the main sites of metastasis are the lung and liver [4]. Likewise, human ACC is typically in advanced stages of disease at presentation, with large tumors and distant metastases being present in approximately 25-50% of cases [39]. Lung, liver and bone are the most common metastatic sites in humans [39]. The molecular mechanisms leading to uncontrolled growth in canine cortisol-secreting ATs are largely unknown.

The activation of the cAMP pathway in both human and canine ATs (**Chapter 3**) [6,14,15], may not only induce ACTH-independent cortisol-secretion, but could also induce uncontrolled adrenocortical growth. In cAMP-sensitive tissue types, such as the adrenal cortex, activating mutations in *GNAS* are known to induce tumor formation by increasing cell proliferation [6,41]. This is thought to result from the downstream activation of several carcinogenesis-related genes and pathways [6,35]. Downstream targets of cAMP/PKA signaling that could be involved in the development of uncontrolled growth include SF-1 [21,22], angiopoietin 2 (*ANGPT2*) [5,42], and the canonical Wnt pathway [6,35].

In the adrenocortical cell, one of the key effectors of ACTH/cAMP signaling is SF-1, well known for its function in adrenal development [43,44]. In the fetal adrenal gland SF-1 stimulates growth independent of steroid synthesis [45,46], and in the adult

adrenal cortex it has a dosage-dependent effect on cell growth [47]. Increased *SF-1* expression induces cell proliferation and decreases apoptosis in human adrenocortical cell lines, and triggers adrenocortical tumorigenesis in mice [48]. In human childhood ACC, increased SF-1 expression is a frequent finding [27]. In the study described in **Chapter 5**, the overall expression of *SF-1* was not increased in cortisol-secreting adenomas or carcinomas, but an increased SF-1 expression was found in the group of carcinomas with recurrence within 2.5 yr [28]. In this group, recurrence was due to metastasis in 6 of the 7 ATs. Based on these observations, a role for *SF-1* expression in cell proliferation and metastasis of canine adrenocortical carcinomas appears likely.

Theoretically, this effect could be mediated by an SF-1 induced increased expression of the angiogenic gene *ANGPT2* [5,42]. Angiogenesis, the process of blood vessel formation from pre-existing vasculature, is important in tumor development as it serves to provide the tumor with the nutrients and oxygen necessary to grow beyond a certain size [49]. Additionally, the newly formed vascular lining is often less stable, facilitating distant metastasis through the extravasation of tumor cells [49]. Therefore, we hypothesized that activation of angiogenesis, possibly through SF-1 induced *ANGPT2* overexpression, could contribute to uncontrolled growth and metastasis of cortisol-secreting ATs. Indeed, in human ACC, high *ANGPT2* expression has been described, and ANGPT signaling has been implicated as a significant factor in the pathogenesis of human ATs [50]. Additionally, human ACC exhibits increased expression of vascular endothelial growth factor (VEGF) which is associated with increased microvascular density [51].

In the study described in **Chapter 6**, a significantly higher *ANGPT2* expression and *ANPGT2/ANGPT1* ratio [52] was detected in both adenomas and carcinomas when compared to normal adrenals [53]. The human H295R cell line was used as a model to evaluate whether *ANGPT2* overexpression could be induced by cAMP signaling, and indeed detected increased *ANGPT2* levels upon cAMP stimulation. Immunohistochemical staining (IHC) showed positive ANGPT2 expression in the tumor cells of all ATs, while ANGPT2 staining in the vascular endothelial lining was limited to carcinomas. The ANGPT2 receptor Tie2 was present exclusively in the tumor vascular endothelium. This could indicate the presence of autocrine or paracrine ANGPT2-Tie loops and consequently a destabilized state of the vascular lining, which facilitates metastasis. Taken together, the results of the study described in **Chapter 6** suggest an *ANGPT2*-induced pro-angiogenic state in canine ATs, possible due to aberrant cAMP/SF-1 signaling, which is likely to be an important contributor to uncontrolled growth and metastasis.

Another effector pathway of cAMP/PKA signaling that is likely to contribute to uncontrolled growth and metastasis is the canonical Wnt pathway. Activation of this pathway, as denoted by nucleo-cytoplasmic β-catenin staining, induces tumor formation by increasing cell survival and cell proliferation. Wnt activation is a frequent event in human ACC [54], frequently caused by somatic activating mutations of the gene encoding β-catenin (*CTNNB1*) [55,56]. Constitutive cAMP/PKA signaling might provide an alternative cause for Wnt activation, as *PRKAR1A* mutations are known to activate Wnt signaling in human ATs by means of increased Wnt ligand expression [6].

In the study described in **Chapter 7**, Wnt-pathway activation was detected in 13 of the 36 canine cortisol-secreting ATs. In 2 of these, activation could be explained by an amino acid changing mutation in *CTNNB1*. Interestingly, these mutations and *GNAS* mutations appeared to be mutually exclusive, which is in accordance with a recent study, demonstrating that in human ATs *CTNNB1*, *GNAS* and *PRKAR1A* mutations were mutually exclusive [9]. In the other ATs, mutations nor increased ligand expression were detected, and the presence of *GNAS* mutations was not associated with a higher Wnt ligand expression. The results of the study described in **Chapter 7** therefore do not support Wnt-activation due to cAMP-induced ligand expression in dogs with an AT. This might be related to the difference between human and canine cAMP-PKA pathway activation, as the effector pathways of *PRKAR1A* and *GNAS* mutations differ, and Wnt-ligands in the latter were lower [35]. Consequently, the cause of Wnt activation in the ATs negative for *CTNNB1* and *AXIN2* mutations remains unclear. However, the results do indicate activation of the Wnt pathway in a large proportion of canine ATs, which is likely to contribute to uncontrolled AT growth.

Based on the results of this PhD study, activation of cAMP/PKA signaling and its downstream targets are likely to contribute to AT growth and metastasis in dogs. However, other, cAMP-independent, mechanisms are also thought to play a role in causing uncontrolled growth in cortisol-secreting ATs. In human ACC, alterations in the insulin-like growth factor (IGF) system, a regulator of adrenocortical cell proliferation, are frequently observed [57,58]. Through the IGF type 1 receptor (IGFR1), IGF signaling activates the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K)/AKT/ mammalian target of rapamycin (mTOR) pathways [59]. In human ACC, overexpression of *IGF-II* [50,60,61], *IGFR1* [62-64] and IGF binding protein 2 (*IGFBP2*) [65], and alterations in the *IGF-II* (11p15) locus [60] are commonly found, and IGF-II is thought to be an autocrine factor in the proliferation of human H295R cells [64]. However, despite these well-documented changes, doubt has risen whether IGF-II overexpression is a driving force for human

adrenocortical tumorigenesis, or merely a passenger alteration [66]. In the study described in **Chapter 8**, a significantly higher expression of *IGF-II* or *IGFR1* was not detected in the canine ATs, and although there was *IGFBP2* overexpression, the significance of *IGFBP2* overexpression in the absence of *IGF-II* overexpression is unknown. Recently, it was proposed that comparative studies in humans and dogs could provide a novel strategy to distinguish driver and passenger alterations in carcinogenesis. Although this was based on genomic amplifications and deletions, the same principle may apply for other study types, and the lack of changes in the IGF system in dogs may strengthen the notion of the IGF system as merely a passenger alteration.

The lack of changes in the IGF system in dogs does not preclude activation of the downstream PI3K/AKT/mTOR pathway, which can also be activated through the epidermal growth factor receptors (*EGFR* and *ERBB2-4*), decreased expression or mutation of phosphatase and tensin homolog (*PTEN*), or mutations of the PI3K catalytic subunit A (*PIK3CA*). Activation of this pathway results in cell proliferation, survival and growth [67], and has been documented in human ACC [68,69]. The results of the study reported in **Chapter 8** indicate PI3K activation in canine adrenocortical carcinomas, but not in adenomas. No amino acid changing mutations were detected in *PTEN* or *PIK3CA*, but the tendency toward a higher *ERBB2* expression in carcinomas might play a role in PI3K activation. Additionally, two of the pathway's target genes, inhibitor of differentiation 1 and 2 (*ID1* and *2*) were expressed at higher levels in the group of carcinomas with recurrence within 2.5 yr. The ID proteins are thought to keep cells in a poorly differentiated, proliferative state [70]. The results therefore suggest that PI3K and/or ID signaling may contribute to the uncontrolled growth of canine ATs. In human ACC, no studies have yet evaluated either *ERBB2* or *ID1* and *2* expression. Considering the recent discussion on the causal role of IGF signaling in human ACC pathogenesis, a renewed evaluation of these PI3K pathway components might be worthwhile.

Therapeutic targets in canine ATs

Currently, the therapeutic options for canine ATs are limited. Complete adrenalectomy is the treatment of choice, provided that no metastases are present at the time of presentation [3]. However, surgery is not always possible or successful and tumor recurrence or metastasis occur regularly [2,4]. Medical management of canine ATs includes the use of mitotane, which selectively destroys adrenocortical cells but may results in severe side effects, and trilostane, which inhibits cortisol secretion but does not affect tumor growth or metastasis. Consequently, there is a

need for the development of new targeted treatments, for which knowledge of the molecular mechanisms behind steroidogenesis and tumor growth is essential. Based on the results of the studies described in this thesis, the genes and pathways with the most potential for future therapeutic development are discussed.

While both our results (**Chapter 3**) [15] and research in human ATs [14] indicate an important role for cAMP/PKA activation in steroidogenesis and growth, targeting this pathway could be difficult, as cAMP is essential as a second messenger in many physiologic processes. Therefore, to implement these findings into therapeutic development, targeting needs to be directed specifically at the AT. As SF-1 is a downstream cAMP effector with a profound influence on steroidogenesis and growth, which is expressed only in steroidogenic tissues and overexpressed in ATs, targeting SF-1 might provide such a strategy. In recent years, different isoquinolinone compounds or SF-1 inverse agonists have been developed, which inhibit SF-1 transcriptional activity [71,72]. Pre-clinical studies showed that SF-1 inverse agonists decrease cell proliferation and cortisol secretion in the human H295R ACC cell line [73]. In dogs, the significantly higher *SF-1* expression in recurrent metastatic ATs, also indicates a therapeutic potential for SF-1 inverse agonists (**Chapter 5**) [28]. Further studies on primary AT cultures of both dogs and humans are needed to explore this therapeutic potential.

Another downstream cAMP effector that could be used in therapeutic development is ANGPT2. In human cancer research, the use of anti-angiogenic drugs is one of the most rapidly emerging therapeutic strategies, and specific inhibitors are available for ANGPT2 and Tie2 [74-76]. In human ATs, despite *ANGPT2* and *VEGF* overexpression and a high vascularity, no studies have yet evaluated the use of angiogenesis inhibitors. In canine ATs, the results of the study described in **Chapter 6** suggest an *ANGPT2*-induced pro-angiogenic state, which is likely to be an important contributor to uncontrolled growth and metastasis [53]. Therefore, targeting of the ANGPT2-Tie2 pathway by selective antagonists such as ANGPT2 traps or monoclonal antibodies [77,78] may hold promise as an adjunctive therapeutic option in ATs.

Because of their anti-secretory and anti-proliferative effects through G-protein inhibitory subunits, and their overexpression in human ATs, targeting SSTRs and DRs could provide another route for AT-specific interference in cAMP signaling. Synthetic long- and short-acting SST and DA analogs are available, which could be used to influence receptor signaling [16,79]. A recent study in the H295R cell line showed decreased cortisol secretion upon treatment with the somatostatin analogue SOM230 [19]. In canine ATs, the presence of mRNA encoding *SSTR1* and *2* and DRD_1 and D_2 in all ATs (**Chapter 4**) does indicate receptor expression, which might be of use in the development of personalized targeted treatment.

Although the results of the study described in **Chapter 7** did not support a link between cAMP/PKA signaling and Wnt activation, an activated Wnt pathway may still be an attractive therapeutic target, as it induces cell proliferation and reduces apoptosis. Therefore, the development of Wnt inhibitors is a hot topic in human cancer research [80]. In human H295R cells, shRNA-induced silencing of β-catenin resulted in decreased cell proliferation and increased apoptosis in vitro and reduced tumor growth in vivo in xenograft studies [56]. Based on the frequent Wnt pathway activation in human and canine ATs, specific inhibitors of the canonical Wnt pathway may be able to play a future role in the targeted AT treatment.

Because of the frequently observed changes in the IGF system in human ACC, several studies have investigated the therapeutic potential of IGF signaling inhibition in humans [39,81]. Thus far, however, the results have been disappointing. Although targeting IGFR1 inhibited proliferation in H295R cells [69,82,83], the beneficial effects in early phase clinical trials have been limited, with low response rates and temporary stable disease as the best response [39]. This has led to the recent notion that IGF alterations might be only passenger alterations [66]. In line with this observation, the results of the study described in **Chapter 8** do not indicate a therapeutic potential for inhibiting IGF signaling in canine cortisol-secreting ATs.

As activation of the PI3K pathway has been indicated in human ACC, and this pathway is a known causal factor in many tumor types, targeting the PI3K pathway may also be a promising strategy. Specific inhibitors for this pathway have been developed on different levels, including EGF receptor, PI3K and mTOR inhibitors [84]. In human H295R cells, dual PI3K/mTOR inhibitors inhibit cell proliferation and hormone secretion [82], but no clinical trials using PI3K inhibitors have been performed yet. The results of the study described in **Chapter 8** suggest activation of the PI3K pathway in carcinomas. The tendency for increased *ERBB2* expression in carcinomas could provide a preclinical rationale for evaluating the effects of single or dual EGF receptor inhibitors in the treatment of canine ATs. Alternatively, downstream pathway inhibition may also be effective. Finally, the overexpression of *ID1* and *2* in recurrent carcinomas could hold implications for therapeutic development, as IDs are thought to keep cells in a poorly differentiated, proliferative state and recent studies have addressed their therapeutic potential [70].

Prognostic markers in canine ATs

In human ACC, several genes and proteins have been identified that are associated with a shorter overall or disease-free survival or with the occurrence of metastases,

and thus can be used as prognostic markers. A microarray study showed that based on unsupervised gene expression clustering, human ACC can be grouped into two prognostic categories [85]. This exemplifies the importance of evaluating gene expression levels in addition to traditional histopathological grading systems. In canine ATs, however, no prognostic markers have been identified yet. Therefore in this PhD study, the relative mRNA expression levels were compared of dogs with recurrent disease within 2.5 yr, mostly due to metastasis, and dogs without recurrent disease. For *SF-1*, *ID1* and *2*, DRD_1 and *IGFBP5*, a significantly higher expression was detected in recurrent carcinomas.

The *SF-1* overexpression in canine carcinomas with recurrent disease (**Chapter 5**) [28] is in accordance with the findings in human adult ACC, where SF-1 staining intensity is negatively correlated with survival and SF-1 is considered a tumor stage-independent prognostic factor [26]. The results suggest that the same might hold true in dogs.

DRD_1 was another factor that was markedly overexpressed in recurrent carcinomas, which may represent an alternative mechanism for cAMP activation (**Chapter 4**). Thus far, no human studies have reported an association between high DRD_1 expression and prognosis in endocrine tumors, therefore more studies are needed to address the significance of this finding.

In human ACC, elements of the IGF axis, in particular *IGF-II*, *IGFR1* and *IGFBP2*, have been identified as independent prognostic markers [64,65,86,87]. In dogs, no differential expression between carcinomas with and without recurrence was detected for these genes (**Chapter 8**). However, a significantly higher *IGFBP5* expression was found in recurrent carcinomas. This has not been reported in human ACC, but is in accordance with studies showing an association between higher *IGFBP5* expression and poor prognosis in other human tumor types, such as mammary and bladder carcinoma.

Finally, the higher expression of *ID1* and *2* in recurrent carcinomas (**Chapter 8**) is also of interest. As ID proteins keep cells in a poorly differentiated, proliferative state, it is not surprising that in human oncology overexpression of both genes is associated with poor prognosis in multiple tumor types [70]. No studies have addressed *ID1* and *2* expression in human ACC, but based on the results in dogs *ID1* and *2* could be promising prognostic markers in canine ATs.

The dog as a model for human ACC

The results of this PhD study have provided new insights into the molecular mechanisms behind steroidogenesis and tumor growth in canine ATs, and have pinpointed several potential therapeutic targets and prognostic markers. The final aim of this thesis was to address the suitability of the dog as a spontaneous, large animal model for human ACC. Here, we compare our results on the molecular pathogenesis of canine ATs with the current knowledge on the molecular pathogenesis of human ACC, and discuss the implications of both similarities and differences for the use of dogs as an ACC model.

A common factor in both human and canine ATs is the aberrant activation of the cAMP pathway (**Chapter 3**), which could cause ACTH-independent cortisol secretion [14,15]. Furthermore, aberrant cAMP activation is known to induce tumorigenesis in cAMP sensitive tissues, and to activate downstream effectors such as *SF-1* [21,22], *ANGPT2* [88] and canonical Wnt signaling [35]. Accordingly, in both human and canine adrenocortical carcinomas (**Chapter 5**), SF-1 expression is associated with a poor prognosis and bears promise as a new therapeutic target [26,28,73]. Likewise, overexpression of *ANGPT2* and activation of the canonical Wnt-pathway are common factors in human and canine ATs (**Chapter 6 and 7**) [50,53,54]. Interestingly, the molecular mechanism behind activated cAMP signaling differs. In humans inactivating mutations of *PRKAR1A* and *PRKACA* are most common, and *GNAS* mutations are rare [6]. In contrast, *GNAS* mutations occur frequently in canine ATs, and no *PRKAR1A* mutations were identified [15]. Mutations in *GNAS* and *PRKAR1A* share several downstream effectors, but other downstream pathways differ according to the origin of cAMP activation [35]. When using canine ATs as a model for human ACC, these differences need to be taken into account.

At first sight, the outcome of studies addressing the IGF signaling pathway in human and canine ATs seems quite different. In human ATs, IGF-II and IGFR1 are frequently overexpressed [89], which is not the case in dogs (**Chapter 8**). However, recent studies have also raised doubt about the causal role of IGF signaling alterations in human ACC [66]. Based on the results described in this thesis, canine and human cortisol-secreting carcinomas both share an activation of the PI3K pathway (**Chapter 8**) [68,69]. Another common factor is the presence of mRNA encoding several hormone receptors in canine and human ATs. The lack of overexpression of *VEGF*, *SSTRs* and *DRs* and the low frequency of *CTNNB1* mutations in canine ATs represent some of the major differences between the findings in canine ATs and those in human ACC. Hypothetically, alterations common to both human and canine ATs could be the ones most likely to have a causal role in AT pathogenesis.

In conclusion, many of the molecular mechanisms involved in AT pathogenesis of humans and dogs are similar or corresponding. Therefore, based on the results of this PhD thesis, canine cortisol-secreting ATs appear to represent a valuable spontaneous large animal model for human ACC. However, some important differences have also been identified, which need to be taken into account when using the dog as a model for human ACC.

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Chapter 10

Summary and conclusions

In dogs, hypercortisolism is one of the most frequently observed endocrine disorders, with an estimated incidence of about 1-2 cases per 1000 dogs per year. Approximately 15% of these cases is due to a cortisol-secreting adrenocortical tumor (AT). Cortisol-secreting ATs are characterized by uncontrolled growth and excessive ACTH-independent cortisol secretion, the molecular mechanisms of which are largely unknown. Treatment options for canine ATs are limited and, thus far, no reliable prognostic markers have been identified. The higher incidence of cortisol-secreting ATs in dogs when compared to humans, raises the question whether the dog might be a good spontaneous animal model for human adrenocortical carcinoma (ACC).

Chapter 3 addresses the molecular mechanisms behind ACTH-independent cortisol secretion. The ACTH/cyclic AMP (cAMP) signaling pathway regulates cortisol secretion in the normal adrenal cortex, and is frequently altered in human cortisol-secreting ATs. Therefore, the presence of alterations in this pathway was evaluated by means of mutation analysis of the genes encoding the melanocortin 2 receptor (*MC2R*), G-protein stimulatory subunit α (*GNAS*) and protein kinase A regulatory subunit 1 A (*PRKAR1A*). No mutations were detected in *MC2R* or *PRKAR1A*, while approximately one third of the ATs carried an activating *GNAS* mutation. This could well explain the ACTH-independent cortisol secretion in the affected subset of ATs and may also play a role in adrenocortical tumorigenesis.

Activation of cAMP signaling may also be due to altered hormone receptor expression, which would represent a unique opportunity for targeted treatment. In human ATs, overexpression of progesterone receptor (PR), growth hormone receptor (GHR), somatostatin receptors (SSTRs) and dopamine receptors (DRs) have been reported. In **Chapter 4** the mRNA expression of these hormone receptors was evaluated in canine ATs. In all ATs and normal adrenals, mRNA encoding *PR*, *GHR*, *SSTR1* and *2* and DRD_1 and D_2 was detected. A lower expression of *SSTR1* and DRD_2 was detected in carcinomas, suggesting tumor escape from the inhibiting influence of these receptors. In carcinomas with recurrence within 2.5 yr, a remarkably higher relative expression of DRD_1 was detected. As DRD_1 activates adenylate cyclase through G-protein stimulatory subunit α, its overexpression may represent an alternative mechanism for cAMP activation.

In **Chapter 5** the mRNA expression and immunohistochemical localization of one of the key effectors of MC2R/cAMP signaling, steroidogenic factor 1 (SF-1), was assessed. In human ACC, high SF-1 expression is associated with a poor prognosis. In all canine ATs and normal adrenals, a positive SF-1 staining was detected. The relative *SF-1* mRNA expression was higher in carcinomas with recurrence within 2.5 yr. Recurrence in these carcinomas was mostly due to metastasis, therefore a role for

SF-1 expression in the autonomous cell growth and metastasis of canine cortisol-secreting carcinomas is likely. Our data provide a preclinical rationale for evaluating the effects of SF-1 inverse agonists on AT cell proliferation and steroidogenesis.

One of the downstream targets of SF-1 signaling is the angiogenesis-related gene angiopoietin 2 (*ANGPT2*). Angiogenesis, the process of new vessel formation from existing vasculature, is essential for tumor growth beyond a certain size, and facilitates tumor cell extravasation. **Chapter 6** addresses the expression of angiogenesis-related genes. In canine ATs, the mRNA expression of *ANGPT2* and the *ANGPT2/ANGPT1* ratio were higher, when compared to normal adrenals. Expression of *ANGPT2* could be induced by cAMP stimulation in human H295R ACC cells. In all ATs, immunohistochemical evidence of ANGPT2 expression was detected in tumor cells, while ANGPT receptor Tie2 was expressed in the vascular endothelial lining. In carcinomas, the vascular endothelial lining was also positive for ANGPT2 staining, indicating the presence of an autocrine ANGPT2-Tie2 loop and a destabilized state of the vasculature, which may facilitate metastasis. Taken together, the results indicate an ANGPT2-induced angiogenic state in canine ATs, possible due to increased cAMP signaling. ANGPT2-induced angiogenesis is likely to contribute to AT growth and metastasis, therefore the use of ANGPT2-specific anti-angiogenic drugs may hold therapeutic benefits.

Another downstream target of cAMP/PKA activation which is likely to contribute to tumor growth and metastasis is the canonical Wnt signaling pathway. In human ACC, activation of this pathway is a common event, frequently due to activating mutations in the gene encoding β-catenin (*CTNNB1*). In **Chapter 7** the activation of the Wnt pathway was evaluated by means of immunohistochemical β-catenin localization. In approximately one third of the ATs, activation of the pathway was detected, as indicated by the presence of nucleo-cytoplasmic β-catenin staining. In 2 ATs, Wnt activation could be attributed to activating mutations in *CTNNB1*. In the ATs negative for these mutations, the cause of activation was unclear, as no increased expression of Wnt-ligands was detected. The presence of *GNAS*, *CTNNB1* and *AXIN2* mutations appeared to be mutually exclusive, and the presence of *GNAS* mutations was not associated with a higher Wnt ligand expression. Therefore, in canine ATs the results do not support Wnt pathway activation due to cAMP-induced ligand expression. However, the activation of the Wnt pathway detected in a substantial portion of ATs very likely contributes to uncontrolled growth and metastasis, and may thus represent an attractive therapeutic target.

Like the canonical Wnt pathway, the phosphatidylinositol 3 kinase (PI3K) pathway induces tumor cell proliferation, and activation of this pathway has been reported in human ACC. The increased expression of insulin-like growth factor 2 (*IGF-II*) and the

type 1 IGF receptor (*IGFR1*) in human ACC has been implicated as a potential cause for PI3K activation. **Chapter 8** addresses the presence of PI3K activation in canine ATs. Target gene expression indicated PI3K activation in carcinomas, which could not be attributed to *IGF-II* or *IGFR1* overexpression or to mutations in phosphatase and tensin homolog (*PTEN*) or PI3K catalytic subunit (*PIK3CA*). The tendency toward a higher *ERBB2* expression in carcinomas might play a role in PI3K activation. In carcinomas with recurrence within 2.5 yr, the relative expression levels of IGF binding protein 5 (*IGFBP5*) and inhibitor of differentiation 1 and 2 (*ID1* and *2*) were significantly higher, and might thus be valuable as prognostic markers. Additionally, *ERBB2*, *ID1* and *ID2* may represent attractive new therapeutic targets.

Chapter 11

Samenvatting en conclusies

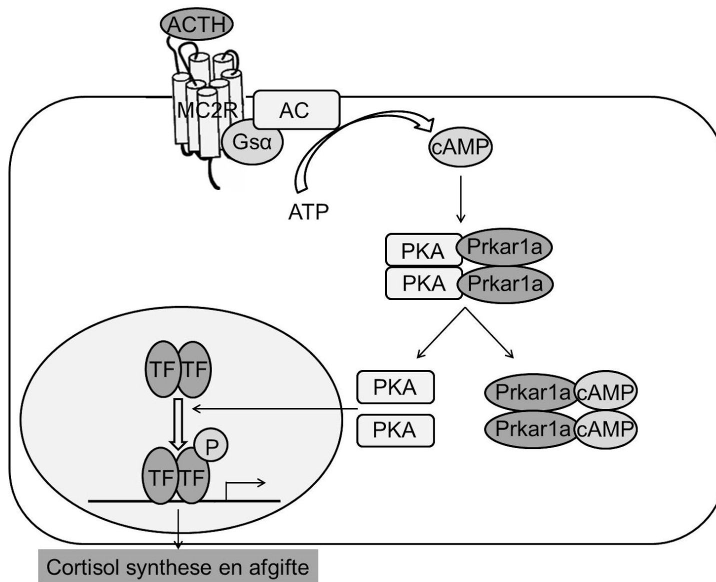
Hypercortisolisme, ofwel het syndroom van Cushing, is een van de meest voorkomende hormonale aandoeningen van de hond. Als gevolg van de te hoge concentratie cortisol in het bloed kunnen honden met hypercortisolisme verschillende klinische verschijnselen vertonen, zoals teveel plassen, teveel drinken, een dunnere huid, kaalheid (in de flanken), een dikke buik, spierafbraak en een verminderde inspanningstolerantie. In ongeveer 15% van de gevallen wordt hypercortisolisme bij de hond veroorzaakt door een cortisol-producerende tumor van de bijnierschors. Bijniertumoren van de hond kunnen zowel goedaardig (adenomen) als kwaadaardig (carcinomen) zijn. De belangrijkste kenmerken van cortisol-producerende bijniertumoren zijn een ongeremde groei en een overmatige afgifte van cortisol, die niet afhankelijk is van de ACTH afgifte door de hypofyse. De moleculaire mechanismen die bijdragen aan deze ACTH-onafhankelijke cortisolafgifte en de ongecontroleerde tumorgroei zijn grotendeels onbekend.

De therapeutische mogelijkheden voor cortisol-producerende bijniertumoren bij de hond zijn beperkt. Een volledige operatieve verwijdering van de bijniertumor (adrenalectomie) heeft de voorkeur, mits op het moment van de diagnose geen uitzaaiingen en geen ingroei in de grote bloedvaten aanwezig zijn. Als adrenalectomie niet mogelijk is of wanneer na adrenalectomie recidief optreedt, is een behandeling met mitotane een goede optie. Mitotane is een chemotherapeuticum dat selectief de cellen van de bijnierschors vernietigt, maar ernstige bijwerkingen kan hebben. Het alternatief is een palliatieve behandeling met trilostane, een remmer van een van de enzymen die betrokken zijn bij de synthese van cortisol. Met trilostane kan de cortisolafgifte worden verminderd, en daarmee ook de symptomen van hypercortisolisme, maar de groei en uitzaaiing van de tumor worden niet beïnvloed. De ontwikkeling van nieuwe medicamenteuze behandelingen voor cortisol-producerende bijniertumoren bij de hond is dus van groot belang. Voor een gerichte ontwikkeling hiervan is een gedegen kennis over de moleculaire mechanismen die verantwoordelijk zijn voor de ACTH-onafhankelijke cortisolafgifte en ongecontroleerde tumorgroei essentieel.

Cortisol-producerende bijniertumoren komen ook bij de mens voor, maar de incidentie bij honden is ongeveer 1000 keer zo hoog als bij de mens. Hierdoor komt de vraag op of honden met spontane bijniertumoren mogelijk een goed model zouden kunnen zijn voor bijniercarcinomen bij de mens. Het is goed mogelijk dat het ophelderen van de moleculaire mechanismen die betrokken zijn bij de cortisolafgifte en groei van bijniertumoren bij de hond, kunnen worden vertaald naar de humane geneeskunde, en kunnen bijdragen aan het onderzoek naar humane bijniercarcinomen.

Hoofdstuk 3 van dit proefschrift beschrijft een onderzoek naar de moleculaire oorzaken van de ACTH-onafhankelijke cortisolafgifte. De productie van cortisol in de normale bijnier wordt in gang gezet doordat ACTH bindt aan haar receptor, de melanocortin 2 receptor (MC2R). In de cel leidt dit vervolgens tot de productie van cyclisch AMP (cAMP), de activering van het eiwit Protein Kinase A (PKA), en de synthese van cortisol (Fig. 1). Cortisol-producerende bijniertumoren van de mens hebben vaak mutaties in de genen die coderen voor eiwitten betrokken bij het activeren van de cortisolsynthese. Daarom is er in het onderzoek dat in dit hoofdstuk wordt beschreven gezocht naar mutaties in de genen die coderen voor *MC2R*, de stimulerende alpha component van het G-eiwit (*GNAS*) en de regulerende component 1A van PKA (*PRKAR1A*). Geen van de bijniertumoren vertoonde een mutatie in *MC2R* of *PRKAR1A*, maar in ongeveer een derde van de bijniertumoren werd een activerende mutatie van *GNAS* gevonden. Deze mutaties zouden door activatie van cAMP/PKA de ACTH-onafhankelijke cortisolafgifte in de aangedane bijniertumoren kunnen verklaren, en zouden daarnaast ook kunnen bijdragen aan de ontwikkeling van de bijniertumoren.

Behalve door de MC2R, kan de productie van cAMP ook worden gestimuleerd door andere hormoonreceptoren, bijvoorbeeld als deze verhoogd tot expressie komen in de bijnierschors. De expressie van deze hormoonreceptoren in het tumorweefsel kan daarnaast een unieke mogelijkheid bieden voor medicamenteuze behandeling. In bijniertumoren van de mens is een verhoogde expressie van de progesteron receptor (*PR*), de groeihormoonreceptor (*GHR*), de somatostatine-receptoren (*SSTRs*) en de dopaminereceptoren (*DRs*) gerapporteerd. In het onderzoek beschreven in **Hoofdstuk 4** is de mate van genexpressie van deze hormoonreceptoren onderzocht in cortisol-producerende bijniertumoren van de hond. In alle bijniertumoren en normale bijnieren was de mRNA expressie van *PR*, *GHR*, *SSTR1* en *SSTR2* en DRD_1 en DRD_2 aanwezig. Carcinomen vertoonden een significant lagere expressie van *SSTR1* en DRD_2. Mogelijk wijst dit op een ontsnappingsmechanisme van de tumor aan de remmende invloed op de groei en hormoonproductie die deze receptoren normaliter uitoefenen. In de groep carcinomen met recidief binnen 2,5 jaar, was de expressie van DRD_1 beduidend hoger dan in de groep carcinomen waarbij geen recidief optrad na de adrenalectomie. Aangezien DRD_1 via de stimulerende alpha component van het G-eiwit de productie van cAMP stimuleert, zou de verhoogde expressie van DRD_1 een alternatief mechanisme voor cAMP/PKA activatie kunnen vormen.



Figuur 1

Schematische weergave van de signaaltransductie die leidt tot cortisolsynthese in de bijniercel.

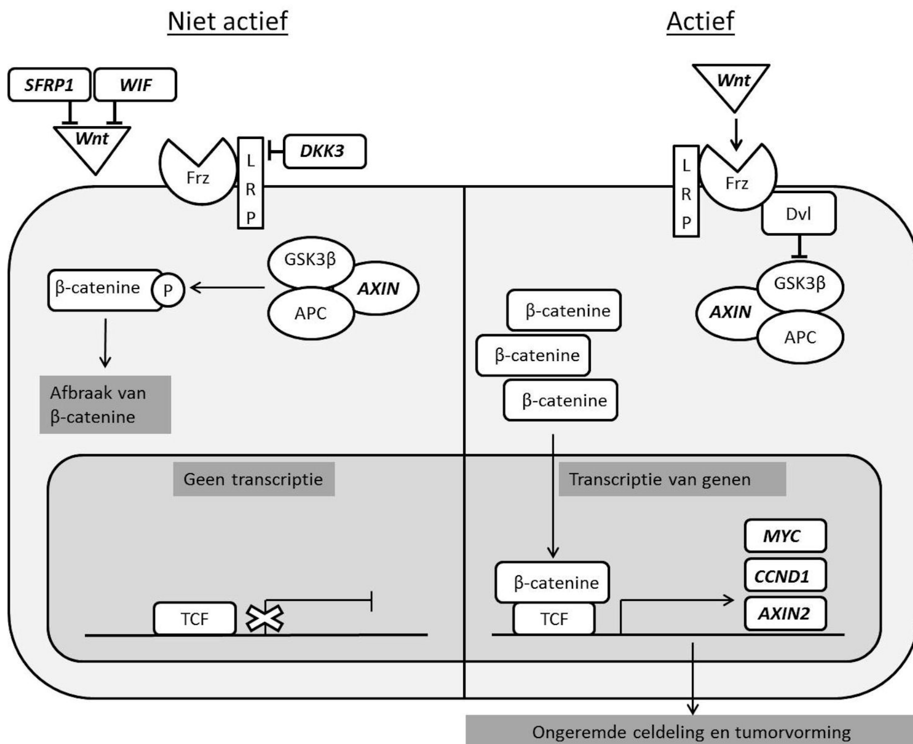
Het binden van het bijnierschors-stimulerend hormoon ACTH aan haar receptor, de melanocortin 2 receptor (MC2R), vormt de eerste stap van de cascade die leidt tot cortisolsynthese. Na de binding aan de receptor wordt in de bijniercel de stimulerende alpha component van het G-eiwit (Gsα) geactiveerd, die het enzym adenylaat cyclase (AC) stimuleert om ATP om te zetten in cAMP. cAMP zorgt ervoor dat het enzym protein kinase A (PKA) wordt losgemaakt van een regulerende component (PRKAR1A), waardoor het enzym PKA actief wordt en zich naar de celkern kan verplaatsen. In de celkern fosforyleert PKA diverse transcriptiefactoren (TF), waardoor deze worden geactiveerd, en kunnen zorgen voor de synthese en afgifte van cortisol.

Een van de belangrijkste mediatoren van ACTH/cAMP signaaltransductie is steroidogenic factor 1 (SF-1). Een te hoge expressie van dit gen leidt bij de muis tot het ontstaan van bijniertumoren, en is in humane bijniercarcinomen geassocieerd met een slechte prognose. In het onderzoek beschreven in **Hoofdstuk 5** werd de expressie van SF-1 geëvalueerd, door middel van een eiwitkleuring en door middel van mRNA expressie analyse. Alle bijniertumoren en normale bijnieren vertoonden een positieve eiwitkleuring voor SF-1. De relatieve mRNA expressie van *SF-1* was hoger in de groep bijniercarcinomen met recidief binnen 2,5 jaar. In 6 van de 7 gevallen was dit recidief het gevolg van een uitzaaiing. Het is daarom waarschijnlijk dat SF-1 een rol speelt bij de ongecontroleerde groei en uitzaaiing van bijniertumoren. Het remmen van de activiteit van SF-1 met behulp van SF-1 inverse agonisten zou daarom een veelbelovende strategie kunnen zijn voor het behandelen van cortisol-producerende bijniertumoren van de hond.

Bij de mens is bekend dat SF-1 de expressie van angiopoietine 2 (*ANGPT2*) stimuleert. Angiopoietine 2 speelt een belangrijke rol bij de angiogenese, het proces waarbij nieuwe bloedvaten worden gevormd vanuit bestaande bloedvaten. Doordat deze nieuwe bloedvaten de toevoer van zuurstof en nutriënten verzorgen, is actieve angiogenese noodzakelijk voor de groei van tumoren. De wand van de nieuwgevormde bloedvaten is vaak minder stabiel, waardoor tumorcellen gemakkelijker kunnen infiltreren in de bloedbaan, en daardoor uitzaaiingen kunnen vormen. In het onderzoek beschreven in **Hoofdstuk 6** werd de expressie van verschillende, bij de angiogenese betrokken genen geëvalueerd. Bijniertumoren van de hond vertoonden een hogere expressie van *ANGPT2*, die gepaard ging met een verhoogde *ANGPT2*/*ANGPT1* ratio, welke indicatief is voor een actieve angiogenese. De expressie van *ANGPT2* kon in humane bijniercarcinoomcellen worden gestimuleerd door de cellen met cAMP te incuberen. Met een eiwitkleuring is gekeken naar de lokalisatie van ANGPT2 en haar receptor Tie2. In alle bijniertumoren werd ANGPT2 gedetecteerd in de tumorcellen, en Tie2 in de bloedvatwanden. In carcinomen werd ANGPT2 daarnaast ook gedetecteerd in de bloedvatwanden. De aanwezigheid van zowel ANGPT2 als Tie2 in de bloedvatwand van carcinomen, kan duiden op een interne terugkoppelingslus en een instabiele vaatwand, wat uitzaaiing kan bevorderen. Tezamen genomen wijzen de resultaten van dit onderzoek op een ANGPT2-geïnduceerde actieve angiogenese, mogelijk ten gevolge van cAMP activatie. Het is waarschijnlijk dat deze actieve angiogenese bijdraagt aan de ongeremde groei en uitzaaiing van bijniertumoren, en daarom zou het gebruik van ANGPT2-specifieke remmers van de angiogenese een waardevolle therapeutische strategie kunnen vormen voor bijniertumoren van de hond.

Behalve de angiogenese kan ook de canonical Wnt cascade door cAMP worden geactiveerd. Wnt activatie (Fig. 2) bevordert de proliferatie van cellen en remt de celdood, en zou daardoor kunnen bijdragen aan de ongeremde groei van bijniertumoren. In humane bijniercarcinomen wordt inderdaad vaak Wnt activatie vastgesteld, welke in een groot deel van de gevallen wordt veroorzaakt door een activerende mutatie in het gen voor β-catenine (*CTNNB1*). In **Hoofdstuk 7** wordt een onderzoek beschreven naar Wnt activatie in cortisol-producerende bijniertumoren van de hond. Gebaseerd op het aantonen van β-catenine kleuring in zowel de celkern als het cytoplasma, was er in ongeveer een derde van de bijniertumoren sprake van Wnt activatie. In 5 van deze tumoren kon de activatie worden toegeschreven aan een mutatie in *CTNNB1* of *AXIN2*. In de tumoren die deze mutaties niet hadden, werd geen verhoogde expressie van Wnt liganden gevonden, en daarmee is de oorzaak van de Wnt activatie bij deze tumoren nog onbekend. De mutaties in *CTNNB1*, *AXIN2* en *GNAS* waren nooit tegelijkertijd in 1 tumor aanwezig, en ook was de expressie van Wnt liganden niet verhoogd in de tumoren met een *GNAS* mutatie. Het is daarom

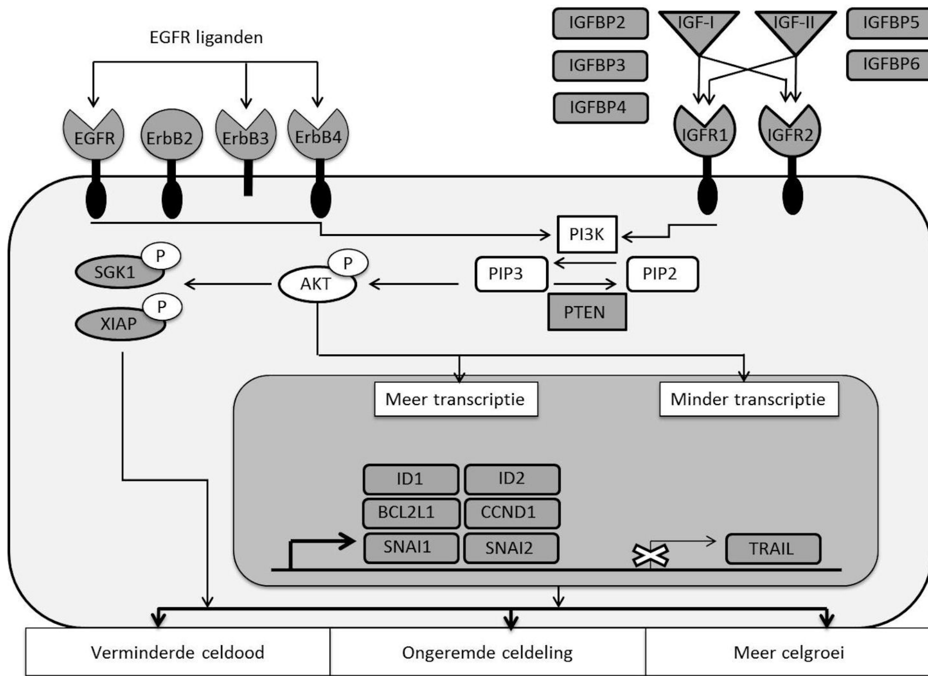
onwaarschijnlijk dat *GNAS* mutaties de Wnt activatie veroorzaken in bijniertumoren van de hond. Echter, de Wnt activatie in een aanzienlijk deel van de tumoren zal zeer waarschijnlijk wel bijdragen aan de ongeremde groei en uitzaaiing van deze tumoren. Daarmee is de Wnt cascade interessant voor de ontwikkeling van nieuwe medicamenteuze therapieën.



Figuur 2

Schematische weergave van de canonical Wnt cascade. Wanneer de Wnt cascade niet actief is (links), binden secreted frizzled related protein 1(SFRP1) en Wnt inhibiting factor (WIF) aan de Wnt eiwitten, en voorkomen daarmee de binding van de eiwitten aan hun receptor frizzled (Frz). Daarnaast remt dickkopf 3 (DKK3) de Wnt cascade door een interactie met co-receptor lipoprotein receptor related protein (LRP). In het cytoplasma van de cel wordt vervolgens β-catenine gefosforyleerd door het complex van Axin, adenomatous polyposis coli (APC) en glycogeen synthase 3 beta (GSK3β). Als gevolg hiervan wordt β-catenine afgebroken voor het zich naar de celkern kan verplaatsen, en vindt er geen transcriptie van de doelgenen plaats.
De Wnt cascade kan op verschillende manieren worden geactiveerd (rechts). Als Wnt eiwitten ongeremd aan de Frz receptor kunnen binden, wordt het Axin-APC-GSK3β complex geremd door dishevelled (Dvl). In het cytoplasma kan β-catenine zich daardoor dan ophopen. Deze ophoping treedt ook op wanneer β-catenine niet kan worden afgebroken, bijvoorbeeld door een mutatie in β-catenine zelf of in de componenten van het Axin-APC-GSK3β complex. Als gevolg van de ophoping in het cytoplasma, kan β-catenine zich vervolgens verplaatsen naar de celkern. Daar bindt het aan de T-cel factor (TCF) en activeert de transcriptie van verschillende genen, zoals cyclin D1 (CCND1), AXIN2 en cMYC (MYC). Het gevolg van Wnt activatie is verminderde celdood, ongeremde celdeling en tumorgroei.

Net als de Wnt cascade, stimuleert ook de phophatidylinositol 3 kinase (PI3K) cascade de groei van tumorcellen (Fig. 3). Bijniertumoren van de mens vertonen regelmatig activatie van deze cascade. De verhoogde expressie van de genen voor insuline-achtige groeifactor 2 (*IGF-II*) en de type 1 IGF receptor (*IGFR1*) in humane bijniercarcinomen zou hiervoor verantwoordelijk kunnen zijn. In **Hoofdstuk 8** wordt een onderzoek beschreven naar PI3K activatie in bijniertumoren van de hond.



Figuur 3

Schematische weergave van de phosphatidylinositol 3 kinase (PI3K) cascade.

Wanneer een ligand bindt aan de epidermale groeifactor (EGF) receptoren (EGFR en ERBB2-4), vormen deze dimeren met elkaar en met zichzelf, waardoor hun kinasedomein actief wordt. De insuline-achtige groeifactor (IGF) receptoren (IGFR1 en 2) worden geactiveerd door binding van IGF-I en II. De acties van IGF-I en IGF-II worden gemoduleerd door de IGF-bindende eiwitten (IGFBP) 2-6. De EGF receptoren en IGFR1 geven hun signalen door via phosphatidylinositol 3 kinase (PI3K). PI3K zet het inactieve PIP2 om in het actieve PIP3. Phosphatase en tensin homoloog (PTEN) remt de PI3K cascade door PIP3 weer naar PIP2 om te zetten. PIP3 zorgt voor een fosforylering van AKT naar P-AKT. In het cytoplasma zorgt P-AKT voor activatie van SGK1 en XIAP door middel van fosforylering. In de celkern activeert P-AKT de transcriptie van ID1 en ID2, SNAI1 en SNAI2, BCL2L1 en CCND1, terwijl het de transcriptie van TRAIL remt. Het gevolg van een geactiveerde PI3K cascade is een verminderde celdood en een ongeremde celdeling.

Afkortingen: SGK1: serum glucocorticoid regulated kinase 1, XIAP: X-linked inhibitor of apoptosis, ID1: inhibitor of differentiation 1, ID2: inhibitor of differentiation 2, SNAI1: snail, SNAI2: slug, BCL2L1: B-cell lymphoma 2 related protein, CCND1: cyclin D1, TRAIL: tumor necrosis factor superfamily member 10

Expressieanalyse van genen die door de PI3K cascade worden geactiveerd, duidde op een activatie van PI3K in carcinomen, die niet kon worden toegeschreven aan een verhoogde expressie van *IGF-II* of *IGFR1*, en ook niet aan mutaties in de genen die coderen voor PI3K remmer phosphatase en tensine homoloog (*PTEN*) of de PI3K katalytische component A (*PIK3CA*). De verhoogde expressie van de epidermale groeifactor-receptor *ERBB2* zou wel een rol kunnen spelen in het ontstaan van PI3K activatie. In de groep bijniercarcinomen met recidief binnen 2,5 jaar was de expressie van de genen voor IGF-bindend eiwit 5 (*IGFBP5*) en voor de remmers van differentiatie 1 en 2 (*ID1* en *2*) significant hoger. Deze genen zouden dus als indicatoren voor de prognose dienst kunnen doen. Bovendien zouden ERBB2, ID1 en ID2 aantrekkelijke doelwitten kunnen zijn voor het ontwikkelen van een medicamenteuze therapie.

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List of abbreviations

17βHSD	17β-hydroxysteroid dehydrogenase
3βHSD	3β-hydroxysteroid dehydrogenase
Ab	antibody
AC	adenylate cyclase
ACC	adrenocortical carcinoma
ACTH	adrenocorticotropic hormone
AMP	adenosine monophosphate
ANGPT	angiopoietin
APC	adenomatous polyposis coli
AT	adrenocortical tumor
ATP	adenosine triphosphate
AVP	arginine-vasopressin
BCL2L1	B-cell lymphoma 2 related protein
bFGF	basic fibroblast growth factor
BP	blocking peptide
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CCND1	Cyclin D1
CRH	corticotropin-releasing hormone
CTNNB1	β-catenin
CYP11A	cytochrome P450 cholesterol side chain cleavage
CYP11B1	11β-hydroxylase type 1
CYP17	17β-hydroxylase/17,20-lyase
CYP21	21-hydroxylase
DA	dopamine
DKK3	Dickkopf 3
DR	dopamine receptor
Dvl	disheveled
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ERBB	erythroblastic leukemia viral oncogene homolog
FAK	focal adhesion kinase
Frz	Frizzled
GDP	guanidine diphosphate
GHR	growth hormone receptor
Gi	G-protein inhibitory subunit
GIP	gastric inhibitory polypeptide

GNAS	stimulatory G protein alpha subunit
GNASL	stimulatory G protein alpha subunit, long variant
GNASS	stimulatory G protein alpha subunit, short variant
GR	glucocorticoid receptor
GSK3β	glycogen synthase 3 β
Gsα	stimulatory G protein alpha subunit
GTP	guanidine triphosphate
GUSB	beta-glucuronidase
HE	haematoxylin and eosin
HPA	hypothalamic-pituitary-adrenal
HPRT	hypoxantine phosphoribosiltransferase
HRP	horseradish peroxidase
ID	inhibitor of differentiation
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
IGFR	insulin-like growth factor receptor
IHC	immunohistochemistry
INSR	insulin receptor
LH	luteinizing hormone
LRP	lipoprotein receptor-related protein
MAPK	mitogen activated protein kinase
MC2R	melanocortin 2 receptor
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
MYC	V-Myc avian myelocytomatosis viral oncogene homolog
NFKB	nuclear factor kappa-B
p-AKT	phosphorylated AKT
PBS	phosphate buffered saline
PDE	phosphodiesterase
PI3K	phosphatidylinositol 3 kinase
PIK3CA	phosphatidylinositol 3 kinase catalytic subunit
PKA	protein kinase A
PPNAD	primary pigmented nodular adrenocortical disease
PR	progesterone receptor
PRKACA	protein kinase A catalytic subunit A
PRKAR1A	PKA regulatory subunit 1A
PTEN	phosphatase and tensin homolog
qPCR	quantitative RT-PCR
RPS19	ribosomal protein S19

RPS5	ribosomal protein S5
RT-PCR	reverse transcriptase polymerase chain reaction
SF-1	steroidogenic factor 1
SFRP1	secreted frizzled related protein 1
SGK1	serum glucocorticoid regulated kinase 1
SNAI1	snail
SNAI2	slug
SNP	single nucleotide polymorphism
SPRP	small proline rich protein
SS	somatostatin
SSTR	somatostatin receptor
StAR	steroidogenic acute regulatory protein
TBP	tata binding protein
TCF	T cell factor
TF	transcription factor
TRAIL	tumor necrosis factor superfamily member 10
UTR	untranslated region
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
WIF	Wnt inhibitory factor
XIAP	X-linked inhibitor of apoptosis
ZF	zona fasciculata
ZG	zona glomerulosa
ZR	zona reticularis

Curriculum Vitae

Miriam Margaretha Jantine Kool was born on June 7th 1981in Wageningen, the Netherlands. She attended the Willem Lodewijk Gymnasium in Groningen and the Zernike Athenaeum in Haren, where she graduated cum laude in 2000. In the same year she started to study veterinary medicine at the Faculty of Veterinary Medicine, Utrecht University, and chose companion animal medicine as a differentiation. In 2007-2008 she did an extended one year research internship at the Department of Clinical Sciences of Companion Animals, under supervision of Dr. Sara Galac, Dr. Hans Kooistra and Dr.Ir. Jan Mol, on the topic of angiogenesis in canine cortisol-secreting adrenocortical tumors.

She graduated as a veterinarian in November 2008, but was so captured by research that together with her supervisors she submitted a proposal for a PhD project on canine adrenocortical tumors to Morris Animal Foundation and Pfizer Animal Health. In July 2009, this proposal was awarded a MAF/PAH veterinary fellowship for advanced study, and in November Miriam could start her PhD project, entitled "Pathogenesis of canine cortisol-secreting adrenocortical tumors".

Together with her partner Erwin Möller, Miriam lives in Driebergen-Rijsenburg, the Netherlands.

Miriam Margaretha Jantine Kool werd op 7 juni 1981 geboren te Wageningen. Ze ging naar het Willem Lodewijk Gymnasium te Groningen, en later naar het Zernike Atheneum te Haren, waar zij in 2000 cum laude haar diploma behaalde. In datzelfde jaar startte zij met haar studie Diergeneeskunde aan de Universiteit Utrecht, en koos voor de afstudeerrichting gezelschapsdieren. In 2007-2008 deed ze een verlengde onderzoeksstage van een jaar bij het Departement Geneeskunde van Gezelschaps-dieren, onder supervisie van Dr. Sara Galac, Dr. Hans Kooistra en Dr.Ir. Jan Mol, met als onderwerp de rol van angiogenese in de pathogenese van cortisol-producerende bijniertumoren van de hond.

In november 2008 studeerde Miriam af als dierenarts, maar ze was zo gegrepen door het onderzoek dat ze samen met haar begeleiders een voorstel indiende voor een Morris Animal Foundation / Pfizer Animal Health beurs voor een promotie-onderzoek naar bijniertumoren bij de hond. In juli 2009 kwam het bericht dat de beurs was toegekend, en in november van dat jaar kon Miriam starten met haar promotieonderzoek, getiteld "Pathogenese van cortisol-producerende bijnier-tumoren van de hond".

Samen met haar partner Erwin Möller woont Miriam in Driebergen-Rijsenburg.

List of publications

2014 **Kool MMJ**, Galac S, van der Helm N, Spandauw CG, Kooistra HS, Mol JA. Expression of somatostatin, dopamine, progesterone and growth hormone receptors in canine cortisol-secreting adrenocortical tumours. The Veterinary Journal 2014, *accepted*

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