PROGNOSTIC FACTORS for outcome of PITUITARY SURGERY in DOGS with corticotroph adenomas

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Prognostic factors for outcome of pituitary surgery in dogs with corticotroph adenomas

Prognostische factoren voor de uitkomst van hypofyse chirurgie bij honden met corticotrofe adenomen

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op dinsdag 2 juni 2015 des middags te 2.30 uur

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Contents

1 / Aims and scope of the thesis

Pituitary-dependent hypercortisolism (PDH), or Cushing's disease is a common endocrinopathy in dogs. It is caused by a pituitary corticotroph adenoma, that secretes adrenocorticotropic hormone (ACTH), which leads to hypercortisolism. Common clinical signs of hypercortisolism are polydipsia, polyuria, polyphagia, panting, abdominal distension, endocrine alopecia, hepatomegaly, muscle weakness and systemic hypertension [1]. The reported incidence of PDH in dogs is 1 to 2 per 1000 dogs/year [2]. PDH in dogs can be treated with medication, radiotherapy or surgery. Medical treatment is directed at elimination of cortisol secretion by the adrenal glands. The disadvantage of medical treatment is possible progression of adenoma growth in the pituitary gland. Surgical treatment, i.e. removal of the pituitary gland including the corticotroph adenoma, with transsphenoidal hypophysectomy, is directed at the tumor itself [3]. Although direct postoperative results are good, long-term recurrences of hypercortisolism do occur in around 25% of dogs [4,5]. Therefore, more research is needed for a better understanding of pituitary tumorigenesis, risk factors for recurrence of hypercortisolism and the identification of possible prognosticators after surgery in dogs with PDH. Collection of data and materials of patients treated surgically for PDH at the Department of Clinical Sciences of Companion Animals of Utrecht University since 1993 has led to an extensive database and biobank of pituitary tissue available for research purposes, that forms the basis of this thesis.

In the general introduction (***Chapter 2***) an overview is given of pituitary morphology and development, clinical signs, diagnosis and treatment of dogs with PDH and Cushing's disease in humans. Also, an overview of the current knowledge of pituitary tumorigenesis is given. A causative role for cancer stem cells has been identified in the development of pituitary adenomas [6], therefore, the putative role of stem cells in the pituitary gland and pituitary adenomas is also described.

Previously, the short-term (<3 years) results of transsphenoidal hypophysectomy in 52 dogs (in 1998) and long-term results in 150 dogs (2005) and in 181 dogs (2007) were reported [4,5,7]. The 1-year estimated survival rate was 84% in the studies of Meij et al. 1998 [4] and Hanson et al. 2005 [5], and 86% in the study of Hanson et al. 2007 [7]. The first study in 52 dogs reported a 1-year disease-free fraction of 92%, the second study reported a 1-year disease free fraction of 88%, whereas the last study of Hanson et al. in 181 dogs, reported a 1-year disease free fraction of 90% [4,5,7]. With the introduction in 2002, of a new medical treatment option with trilostane, which has less side effects than mitotane, the selection of dogs as candidates for surgical treatment has changed over the last years. Dogs with a non-enlarged pituitary tend to be treated medically first, whereas the dogs with an enlarged pituitary are frequently referred for surgery. Therefore, it is hypothesized that also the survival of patients after surgery might have changed. In ***Chapter 3***, the long-term follow-up, disease-free fraction and relation between pituitary

size and long-term results in 306 dogs with PDH over a 20-year period are analyzed and the influence of pituitary size on patient selection and successful surgical outcome is studied.

Recurrence of hypercortisolism after surgery in dogs with PDH is a well-recognized problem, indicating the need for reliable prognosticators [7]. In this thesis, both biochemical and biomolecular prognosticators are investigated. In human endocrinology, peri-operative hormone measurements are used to predict recurrences, although no specific tests exist that identify all future recurrences [8]. It is hypothesized that peri-operative hormone values are also useful as prognosticators for outcome after surgery in dogs with PDH. In ***Chapter 4*** the peri-operative measurement of ACTH and cortisol in dogs with PDH and their relation to long-term follow-up data are investigated.

To identify possible molecular prognostic factors for outcome after pituitary surgery, a thorough understanding of pituitary development and tumorigenesis is essential. In the last decade, the pathogenesis of pituitary adenomas has been extensively studied in humans, and to a lesser degree in dogs, and tumor oncogenesis has been studied in knock-out mice [9]. Transcription factors that are identified as essential in pituitary development may also be relevant in the process of tumorigenesis since both show resemblances, e.g., cell division and proliferation. Therefore these transcription factors may also be useful as prognostic markers for outcome after pituitary surgery in dogs. ***Chapters 5*** and ***6*** focus on the expression of such potential prognosticators. In humans, proliferation markers are widely used to study pituitary tumor behavior and to predict surgical outcome. In dogs, pituitary adenomas are divided in enlarged pituitaries and non-enlarged pituitaries by their pituitary height/brain area (P/B) ratio. Dogs with an enlarged pituitary have a less favorable prognosis than dogs with a non-enlarged pituitary [7]. It is hypothesized that the different outcome after surgery for dogs with enlarged or non-enlarged pituitaries is caused by a different expression of proliferation markers, and that these markers can be used as prognosticators. The expression of the proliferation markers Ki-67 and proliferating cell nuclear antigen (PCNA) and the cell cycle inhibitor p27kip1 in canine pituitary corticotroph adenomas and normal pituitary tissue is studied with immunohistochemistry in ***Chapter 5***. Where most corticotroph pituitary adenomas arise from the anterior lobe of the pituitary gland, a small proportion originates in the intermediate lobe. Since intermediate lobe tumors tend to be larger, they are thought to have a worse prognosis [7]. Possibly, transcription factors specific for the intermediate lobe cell fate can be used as prognosticators for surgical outcome. Also, it is thought that stem cells play a role in pituitary tumorigenesis. So-called cancer stem cells (CSCs) are thought to drive the growth and development of a tumor [6]. In this respect, ***Chapter 6*** explores the expression of the melanotroph specific transcription factor Paired box protein 7 (Pax7) and the possible stem cell marker sex determining region Y-box 2 (Sox2)

with immunohistochemistry and qPCR in canine pituitary corticotroph adenomas and normal pituitary tissue.

For reliable interpretation of qPCR results, tissue specific reference genes need to be included and evaluated for their expression stability in experiments. The expression of reference genes in pituitary tissue has not been described before and could be different than in other canine tissues. The expression stability of six frequently used reference genes in pituitary adenoma tissue and normal pituitary tissue of humans, mice and dogs is evaluated in ***Chapter 7***.

Stem and progenitor cells are thought to play a role in pituitary tumorigenesis, but lack of unique stem cell markers hampers the identification and isolation of these cells [6,10]. Understanding of pituitary stem cell biology will contribute to understanding of pituitary pathobiology and will help with identification of possible prognosticators. A promising technique to isolate potential pituitary stem cells is the use of fluorescence activated cell sorting (FACS) to isolate a side population of cells with stem cells characteristics. After identification of this side population in the murine pituitary [11], it was hypothesized that also the canine pituitary gland contains a side population, enriched in cells with stem cell characteristics. ***Chapter 8*** describes the isolation and characterization of the side population in six healthy canine pituitaries.

Chapter 9 gives a summarizing discussion and conclusions.

Aims of the thesis

Clinical parameters

- The analysis of long term follow-up (survival and disease-free fractions) and relation between pituitary size and long-term results in dogs with PDH.
- The analysis of peri-operative plasma ACTH and cortisol concentrations in dogs with PDH and their relation to long-term follow-up data.

Molecular parameters

- The expression of the proliferation markers Ki-67, PCNA and the cell cycle inhibitor p27kip1 in canine pituitary corticotroph adenomas and normal pituitary tissue.
- The expression of Pax7 and Sox2 in canine pituitary corticotroph adenomas and normal pituitary tissue.
- The expression stability of six frequently used reference genes in pituitary adenoma tissue and normal pituitary tissue of humans, mice and dogs.
- The isolation and characterization of the side population in six healthy canine pituitaries.

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2/ General Introduction

Part of this chapter has been published as:

Stem cells in the canine pituitary gland and in pituitary adenomas
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Morphology and function of the pituitary gland

The pituitary gland is a small endocrine gland located in the pituitary fossa, a depression in the basisphenoid bone (Figure 1A). It is rostrocaudally placed with its long axis parallel to the ventral surface of the brain. Rostrally, the fossa is lined with the tuberculum sellae; the dorsum sellae forms its caudal lining [1]. A cylindrical stalk, the infundibulum or pars proximalis neurohypophysis, connects the pituitary gland with the hypothalamus. The third ventricle forms an invagination in the pituitary stalk, the infundibular recess. The pars proximalis neurohypophysis continues into the largest part of the neurohypophysis, the pars distalis neurohypophysis or lobus nervosus. The largest part of the pars distalis adenohypophysis is placed ventrorostrally of the pars distalis neurohypophysis and surrounds it almost totally. The inner part of the adenohypophysis, the pars intermedia adenohypophysis, contacts the pars distalis neurohypophysis directly. Together they form the neurointermediate lobe. Rathke's cleft divides the pars distalis adenohypophysis and the pars intermedia. The pars infundibularis adenohypophysis surrounds the pars proximalis neurohypophysis and covers part of the median eminence. The pituitary gland consists of three functional units: the anterior pituitary, formed by the pars infundibularis and pars distalis of the adenohypophysis; the intermediate lobe (pars intermedia) and the posterior pituitary (neurohypophysis) (Figure 1B) [1,2].

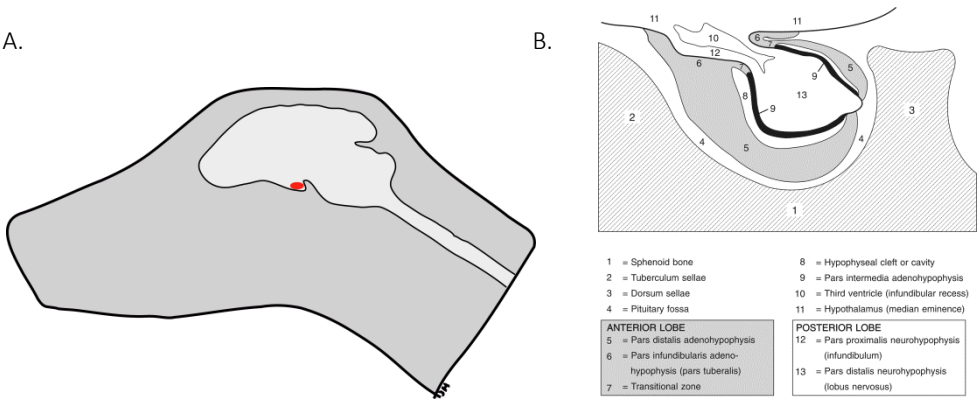

Figure 1. Anatomy of the canine pituitary gland. (A) Schematic lateral view of a canine skull. The pituitary gland (red) is positioned in the cranial cavity underneath the brain. (B) The pituitary gland is lying in the pituitary fossa, which is a shallow hollow in the sphenoid bone. The schematic drawing shows the two parts of the pituitary gland; the adenohypophysis (grey and black) and the neurohypophysis (white) and their respective sub-regions (reproduced with permission, Hanson 2007).

The hypothalamus – pituitary axis plays a central role in maintaining homeostatic functions, like control of metabolism, reproduction, and growth [3]. The anterior pituitary contains five distinct types of endocrine cells (Figure 2); the corticotroph cells that produce adrenocorticotropic hormone (ACTH) and ß-lipoprotein (ß-LPH) account for 10-20% of the total cell population, the thyrotroph cells that produce thyroid stimulating hormone (TSH) comprise 10%, the gonadotroph cells that produce luteinizing hormone (LH) and follicle stimulating hormone (FSH) also represent 10%. Around fifty percent of the cells are somatotroph cells that produce growth hormone (GH). The lactotroph cells that produce prolactin (PRL) make up for 10-20% of the cell population. A small population of cells produces GH and PRL, the mammosomatotroph cells. The anterior pituitary also contains a non-hormone producing population, the folliculostellate cells [4].

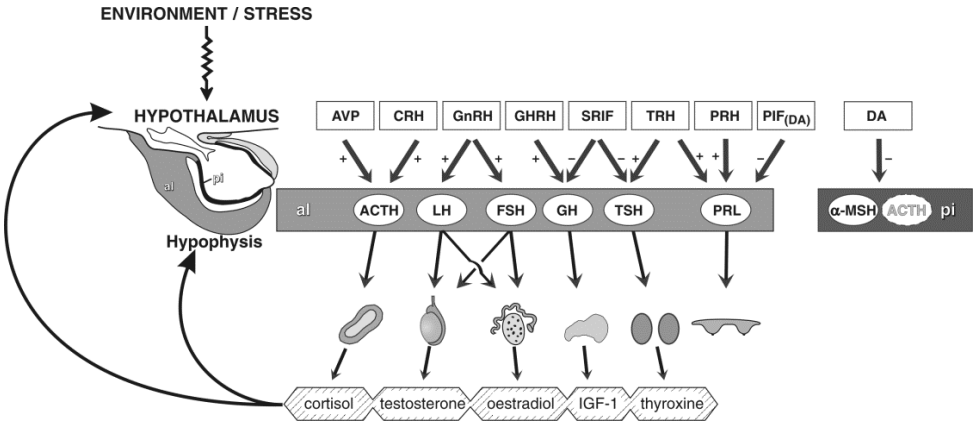

Figure 2. Hypothalamo-pituitary axis. Simplified diagram of hypophysiotrophic regulation of the secretion of hormones in the adenohypophysis. al: anterior lobe adenohypophysis; pi: pars intermedia adenohypophysis; AVP: arginine vasopressine; CRH corticotropin releasing hormone; GnRH: Gonadotropin releasing hormone; GHRH: growth hormone releasing hormone; SRIF: somatotropin-release inhibiting factor or somatostatin; TRH: thyrotropin releasing hormone; PRF: prolactin releasing factor; PIF: prolactin inhibiting factor; DA: dopamine; ACTH: adrenocorticotropic hormone; LH: luteinizing hormone; FSH: follicle-stimulating hormone; GH: growth hormone; TSH: thyroid-stimulating hormone; PRL: prolactin; α-MSH: α-melanocyte-stimulating hormone; IGF-1: insulin-like growth-factor 1 (reproduced with permission, Hanson 2007).

The pars intermedia contains corticotrophs and melanotrophs that produce ACTH and α-melanocyte stimulating hormone (α-MSH), respectively [5]. The neurohypophysis is the storage place for vasopressin (AVP) and oxytocin that are produced in hypothalamic nuclei

[2]. The secretion of this wide variety of hormones by the pituitary gland is under strong influence of different environmental conditions. The pituitary gland responds quickly upon changes in body homeostasis, indicating the cellular plasticity of the pituitary gland.

Development of the pituitary gland

The embryological development of the pituitary is more or less similar for all mammals (Figure 3A). During pituitary organogenesis, the adenohypophysis and neurohypophysis develop from an intimate interaction between the ectoderm of the dorsal aspect of the primitive mouth and the ventral neuroectoderm of the diencephalon, the primordium of the ventral hypothalamus [6-8]. At the site of apposition of these two ectodermal layers, there is a thickening of the ectoderm of the primitive mouth, which then evaginates and forms Rathke's pouch. At the same time, the diencephalon thickens and evaginates to form the infundibulum, which gradually expands and develops into the neurohypophysis. The pars intermedia develops from the point of fusion of the two ectodermal layers; the bulk of the adenohypophysis develops through proliferation of the anterior wall of Rathke's pouch. During organogenesis, progenitor cells are present along the wall of Rathke's pouch that differentiate into the various pituitary cell lineages [9]. With the growth of the embryo Rathke's pouch is closed, the ectodermal stalk regresses and the separation between the adenohypophysis and the oronasal cavity is completed by the formation of the basisphenoid bone [10]. In the adult canine pituitary, Rathke's pouch only persists as Rathke's cleft [10].

There are three main pathways of differentiation for the hormone producing cells in the anterior hypophysis (Figure 3B) [10,11]. Expression of T-pit that binds with corticotropin upstream transcription-binding element (CUTE) proteins determines the differentiation of the pro-opiomelanocortin (POMC) cell lineage, which consists of corticotroph and melanotroph cells [12-14]. Gonadotroph cells need expression of steroidogenic factor 1 (SF-1), GATA-2, and LIM homeobox 4 (Lhx4) [15-17]. Expression of the pituitary transcription factor 1 (Pit-1) and the Prophet of Pit-1 (Prop-1) leads to the development of somatotroph, mammosomatotroph, lactotroph, and thyrotroph cells [17,18].

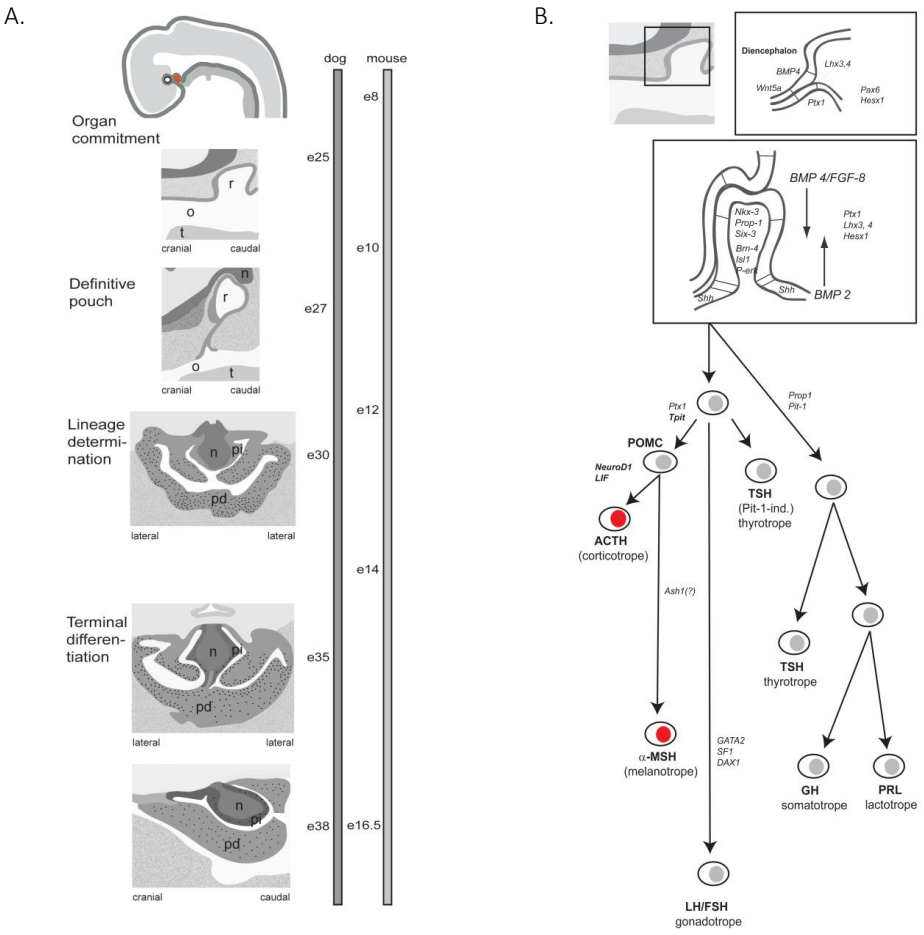

Figure 3. The development of the pituitary gland. (A) Pituitary ontogeny of the canine pituitary gland and the corticotroph cells (black spots in the pars distalis adenohypophysis and darkening of the pars intermedia adenohypophysis) (Adapted from Sasaki and Nishioka 1998). r: Rathke's pouch (the proposed location of stem cells in the postnatal pituitary gland); o: oral cavity; t: tongue; n: neurohypophysis; pi: pars intermedia adenohypophysis; pd: pars distalis adenohypophysis. Right side: Time bar showing embryonic (e) days of development in the dog and mouse. (B) Expression of transcription factors and cellular differentiation during the development of the pituitary gland. Top diagram shows early placode formation and regions of different expression of transcription factors. The middle diagram shows dorsal and ventral transcription factors that build up concentration gradients in which the cells of Rathke's pouch further differentiate. Lower diagram shows differentiation of the hormone-producing cells of the adenohypophysis in relation to embryonic development. Important transcription factors for the respective cell lineages are indicated beside the arrow. (Adapted from [19,20] (reproduced with permission, Hanson 2007).

The POMC cell lineage develops into corticotroph cells that produce ACTH, located in both the anterior lobe and the intermediate lobe of the pituitary gland, and into melanotroph cells that are located in the intermediate lobe and produce α-MSH [13]. It was shown that although Tpit controls terminal differentiation of both lineages, expression of the transcription factor paired box 7 (Pax7) is essential in differentiation into the melanotroph cell lineage [21]. In corticotrophs, the prohormone POMC is processed in ACTH by the peptide prohormone convertase 1 (PC1). In melanotrophs, PC1 cleaves POMC in ACTH, followed by cleavage of ACTH into α-MSH by prohormone convertase 2 (PC2) [21].

The hypothalamic-pituitary-adrenocortical axis

Secretion of cortisol by the adrenal glands is under control of a complex of direct regulations and feedback mechanisms between the hypothalamus, the pituitary gland and the adrenal glands, called the hypothalamic-pituitary-adrenocortical axis. Under influence of corticotropin releasing hormone (CRH) secretion by the hypothalamus, the pituitary secretes ACTH, the main pituitary hormone regulating adrenal steroid secretion. ACTH secretion is influenced by several neuroendocrine mechanisms: episodic secretion, response to stress, feedback inhibition of cortisol and immunological factors [3].

ACTH is secreted in a pulsatile fashion, ranging from 6 to 12 ACTH bursts per 24h in dogs [22]. There seems to be no circadian rhythm in cortisol secretion in dogs, but cortisol secretion does follow the pulsatile pattern of ACTH secretion [22]. ACTH and cortisol are secreted shortly after stress responses in the central nervous system increase the release of CRH and AVP [23,24]. The major inhibitor of ACTH secretion is negative feedback from cortisol. The plasma half-life of ACTH is around 20 minutes in both humans and dogs [25,26], the half-life of cortisol is longer, around 50 minutes in humans [27].

When circulating glucocorticoid concentrations are elevated (hypercortisolism) they cause a complex of physical and biochemical changes which is called Cushing's syndrome after Harvey Cushing, the pioneer of neurosurgery in humans, who, in 1932, first described the syndrome. In dogs, in 15-20% of cases of hypercortisolism this is the result of excessive cortisol secretion by adrenocortical tumors, whereas the remaining 80-85% is caused by an ACTH secreting adenoma in the pars distalis or pars intermedia of the pituitary gland [3]. The estimated incidence of pituitary-dependent hypercortisolism (PDH) is 1 or 2 in 1000 dogs/year [28].

Canine pituitary-dependent hypercortisolism (Cushing's disease)

Clinical signs

PDH is a disease of middle-aged and older dogs, without a gender predilection or breed predisposition. Small breeds tend to be overrepresented. The clinical signs are caused by ACTH-induced hypercortisolism leading to gluconeogenesis and lipogenesis with protein degradation. Common clinical signs of hypercortisolism are polydipsia, polyuria, polyphagia, panting, abdominal distension, endocrine alopecia, hepatomegaly, muscle weakness and systemic hypertension (Figure 4). Less common signs include lethargy, hyperpigmentation, comedones, thin skin, poor hair regrowth, urine leakage and insulin-resistant diabetes mellitus. Uncommon signs are thromboembolism, ligament rupture, facial nerve palsy, pseudomyotonia, testicular atrophy and persistent anestrus [3,29]. Signs associated with local tumor invasion are not common, they only occur when the pituitary tumor is that large that it causes neurological signs [30].

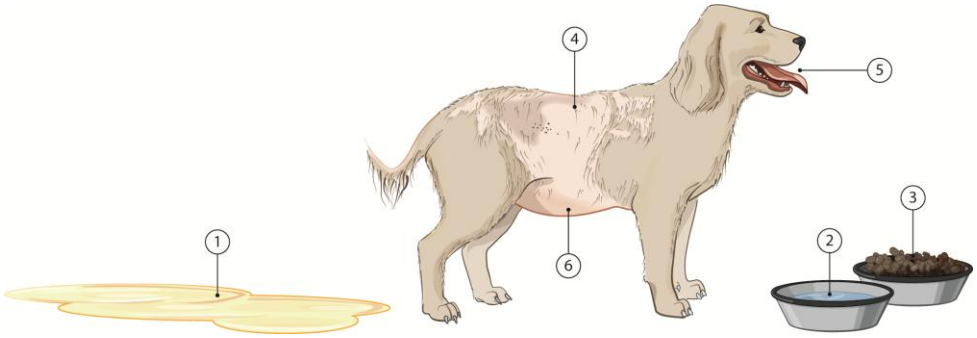

Figure 4. Cushingoid habitus in the dog, characterized by the following clinical signs: polyuria (1), polydipsia (2), polyphagia (3), skin changes (4), panting (5) and pot-bellied appearance (6). (Reproduced with permission, van Deijk 2014).

Diagnosis

The diagnosis of hypercortisolism is based on history, clinical signs and endocrine tests. Endocrine tests should only be performed when clinical signs consistent with hypercortisolism are present. The tests used most often for diagnosis of hypercortisolism include the low dose dexamethasone suppression test, the ACTH stimulation test and the urinary corticoid-to-creatinine ratios (UCCRs) [29]. The last is most commonly used in the Netherlands. In this test, diagnosis is based upon elevated levels of UCCRs in two consecutive morning urine samples collected at home [31,32]. PDH and pituitary-

independent hypercortisolism are distinguished with an oral high-dose dexamethasone suppression test. After collection of the second urine sample, three oral doses of 0.1 mg dexamethasone per kg bodyweight are administered at 6-8h intervals and the next morning a third urine sample is collected. When the UCCR in the third sample is less than 50% of the mean of the first 2 samples, the dog is categorized as being responsive to dexamethasone suppression, and PDH is diagnosed. In cases of dexamethasone-resistant PDH, the diagnosis is confirmed by measurement of the plasma ACTH concentration and further supported by visualization of the adrenals by ultrasonography and pituitary imaging with computed tomography (CT) or magnetic resonance imaging (MRI) [33]. Contrast enhanced pituitary imaging enables visualization of pituitary morphology and pituitary dimensions, since the pituitary is located outside the blood-brain barrier which delineates pituitary tissue nicely from surrounding brain structures [2]. Pituitary lesions range from small nests of hyperplastic cells to large tumors [34]. Pituitary dimensions can be measured on CT and MRI with a resolution of 0.1 mm and in dogs the pituitary height to brain area ratio (P/B ratio) is used to distinguish enlarged pituitaries (P/B ratio >0.31) from non-enlarged pituitaries (P/B ratio ≤ 0.31) [35]. However, diagnosis of PDH cannot solely rely on pituitary imaging, because a normal-sized pituitary does not exclude the presence of a microadenoma [33]. Where an enlarged pituitary gland is readily diagnosed on CT or MRI images, a microadenoma in a non-enlarged pituitary is usually difficult to distinguish from normal pituitary tissue [36]. In these cases, the adenoma can be visualized indirectly by the displacement of the 'pituitary flush' (contrast enhancement of the neurohypophysis) on contrast-enhanced pituitary CT or MRI imaging [36]. Pituitary imaging with CT is also essential in planning of surgical treatment or radiotherapy. CT allows accurate identification of bone surgical landmarks in relation to the pituitary tumor that are required to precisely plan the position of the burr hole for hypophysectomy. For radiotherapy of pituitary tumors with a linear accelerator a CT scout study of the skull is needed to plan the field of radiation inside the skull targeted on the pituitary tumor tissue.

Treatment

PDH in dogs can be treated with medication, radiotherapy or surgery. Medical treatment is directed at elimination of cortisol secretion by the adrenal glands. Until 2002, the most common treatment consisted of selective destruction of the cortex of the adrenal glands with o,p'-DDD (Mitotane®). However, around 30% of dogs suffered from side-effects such as anorexia and vomiting and relapses of hypercortisolism occur frequently. In a study of den Hartog et al. (1999) in 110 dogs, the estimated 1-year disease-free fraction was 77 % and the estimated 1-year survival fraction was 80 % [37].

Since the introduction of trilostane (Vetoryl®) in the Netherlands in 2002, o,p'-DDD is rarely used anymore in the treatment of PDH. Trilostane is a competitive inhibitor of 3β-hydroxy-steroid dehydrogenase which interrupts the synthesis of the hormone cortisol [3]. Survival times for dogs treated with o,p'-DDD and trilostane are similar, but there are less side effects in trilostane treatment and the drug is safe for dog and owner [38-40].

The disadvantage of medical treatment is possible progression of adenoma growth in the pituitary gland. It may be hypothesized that in dogs with PDH the elevated glucocorticoid levels still have a (partial) negative feedback on the pituitary gland, thereby inhibiting some pituitary adenoma growth. With directing the medical treatment to cortisol secretion in the adrenal glands, a higher growth rate could be induced due to elimination of negative feedback. This was seen in a study in normal dogs, where the pituitary gland increased in size during trilostane treatment [41]. A similar mechanism is reported in human patients, after bilateral adrenalectomy in the treatment for CD, they develop Nelson's syndrome [42,43]. This disease is characterized by rapid growth and aggressive transformation of the pituitary adenoma to an invasive adenoma and pituitary carcinoma, and elevated concentrations of ACTH. Indeed, ACTH concentrations rise in dogs with PDH that are treated with trilostane [38]. However, there are no studies available yet that address the development of pituitary dimensions in dogs with PDH under long term trilostane treatment.

Radiation therapy and surgery address the pituitary lesion itself. Radiation therapy reduces the size of the pituitary tumor mass and thereby reduces neurological signs, if present [44-46]. Side effects of pituitary radiation are usually mild and transient [46]. Mean survival time is reported to be 688 to 1,405 days, with an estimated 1-year survival of 93% in the latter study [44,45]. Half the dogs in the study of de Fornel et al. (2007), suffered from recurrence of neurological signs [44]. Although pituitary size may be arrested or reduced, hormone secretion is not immediately altered by pituitary radiation, so dogs with PDH will need additional medical therapy to treat the signs due to glucocorticoid excess [44-46].

Transsphenoidal hypophysectomy is a microsurgical technique that aims to remove the complete pituitary tumor mass and eliminates both neurological and endocrine signs (Figure 5) [1,47]. The 1-, 2-, and 3-year survival fractions are reported to be 84%, 75%, and 72% [48]. However, long term recurrence of clinical signs occurs in 23% of cases that showed initial remission [48]. Dogs treated surgically will need lifelong hormone replacement with cortisone acetate and thyroxine and temporary administration of desmopressin, a vasopressin analogue [1,3,48].

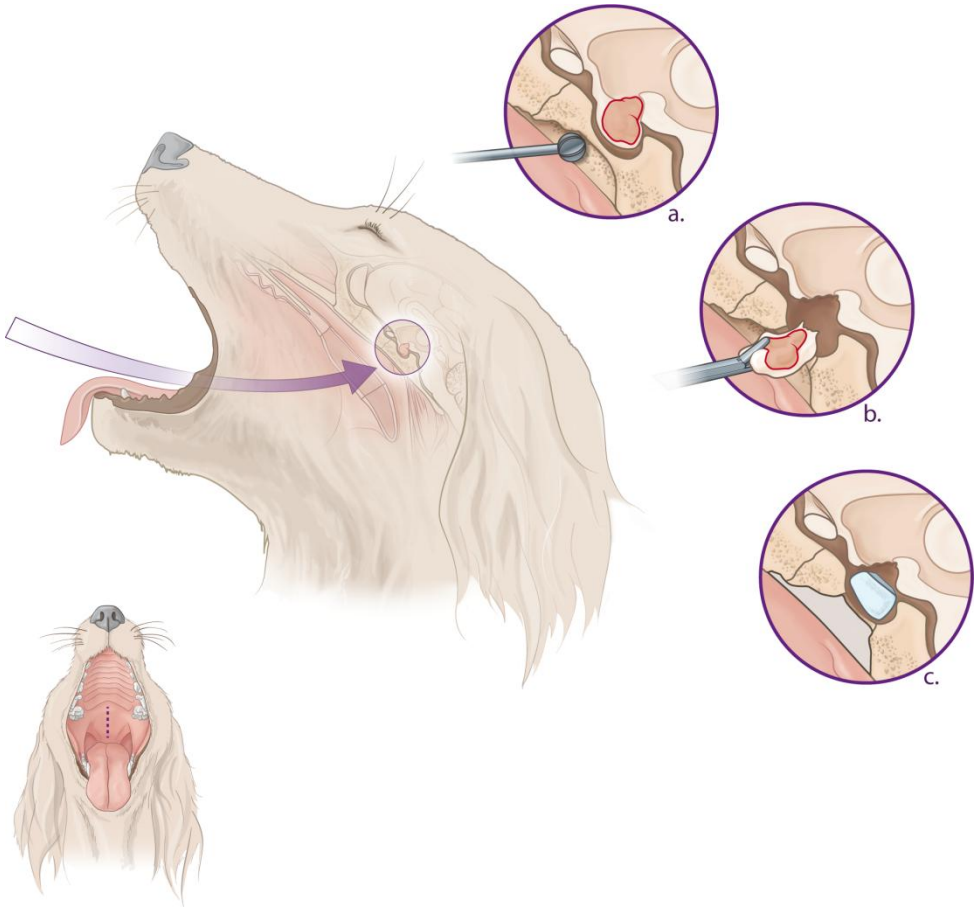

Figure 5. Transsphenoidal hypophysectomy. The pituitary gland is approached through the oropharynx and nasopharynx. The soft palate is incised (----). A burr hole is made (a) in the skull base bone giving access to the pituitary gland with the tumor. The pituitary tumor is removed (b) and the burr hole is filled with a small resorbable gelatin sponge. The hole in the bone is closed with bone wax (c) and the soft palate is sutured (Reproduced with permission, van Deijk 2014).

Cushing's disease in humans

Also in humans, 80% of cases of Cushing's syndrome are caused by pituitary corticotroph adenomas, and is then called Cushing's disease (CD) [49]. Pituitary adenomas are common in human endocrinology, with an estimated prevalence of 1 in 1000. A significant portion of pituitary adenomas in humans are incidental findings on routine imaging of the skull for various reasons [50-53]. Recently, the prevalence of pituitary adenomas in Sweden was studied, with the incidence rate of corticotroph adenomas calculated to be 0.18/100,000, which is consistent with 4% of all the pituitary adenomas that were found. Non-functioning pituitary adenoma were the most common encountered adenomas in this study (54%), followed by prolactinomas (32%), somatotroph adenomas (9%), and TSH-producing pituitary adenomas (0.7%) [53]. Next to functional, ACTH-producing corticotroph adenomas, also silent corticotroph adenomas without excessive hormone production, are found [54].

The diagnosis of CD in humans is complicated by the nonspecificity of clinical signs and includes a variety of biochemical tests and diagnostic imaging techniques [49,55]. The initial treatment of choice for CD is selective pituitary adenomectomy [55]. As in dogs, initial postoperative remission rates are high (65-90%), but long-term recurrences do occur with 5-10% at 5 years after surgery and 10-20% at 10 years after surgery [55]. Other treatment options include hemihypophysectomy or complete hypophysectomy, radiation therapy, medical therapy, and bilateral adrenalecotmy [55].

In both humans and dogs with CD, pituitary surgery is the only tumor targeted therapy with a high long-term success rate. However, medical therapy acting directly on the pituitary lesion, normalizing ACTH secretion and inhibiting tumor growth would mean an enormous advancement in the treatment possibilities for pituitary corticotroph adenomas. These advancements have been very successful in humans in the treatment of prolactinomas and somatotroph adenomas in which medical treatment directed at the pituitary level normalizes PRL and GH/IGF-1 levels, respectively [56,57]. Although multiple pituitary directed therapies have been tested for corticotroph adenomas, such as somatostatine receptor ligands or dopamine D2 receptor agonists [58-61], these therapies are mostly unsuccessful so far. To identify possible new therapeutic targets, a thorough understanding of pituitary development and tumorigenesis is essential and pituitary research concentrates in these fields.

Pituitary tumorigenesis

The study of pituitary tumor pathogenesis has been challenged by several limitations including the inaccessibility of the gland for biopsy material and lack of functional hormone-secreting human pituitary cell lines [62], making research dependent on available patient material and information from transgenic mouse models. Several mechanisms are thought to play a role in pituitary tumorigenesis, including cell cycle regulation, disruption of signaling pathways that play a role in pituitary development, modulation of receptors and the role of stem cells or cancer stem cells [54,63]. Classic oncogene mutations are rarely encountered in pituitary tumors [64], but pituitary-specific cellular disruptions have been discovered [54,65,66].

Cell cycle regulation

Pituitary tumors in humans are monoclonal benign adenomas, rather than hyperplastic growths, which suggests that they in fact arise from expansion of single precursor cells that possess a unique proliferative advantage [67]. The various phases of the cell cycle are regulated by a complex network of cyclins and cyclin-dependent kinases (CDKs). Activated kinases inactivate the tumor suppressor gene retinoblastoma-associated protein (Rb), that restrains cell cycle progression through E2F transcription factor binding [68]. Mice with heterozygous Rb1 inactivation develop pituitary tumors [69] and cyclins are overexpressed in nonfunctioning pituitary adenomas [70], but the exact mechanism for cell cycle disruption remains unclear. Disruption of tumor suppressor genes in two families of CDK inhibitors, p18 and p27, leads to the development of pituitary hyperplasia and adenoma in mouse models [71-73]. One of the downregulators of p27 is epidermal growth factor (EGF), which is expressed in pituitary adenomas [74]. Mutations in the gene coding for p53, that blocks cell cycle progression from G1, are related to tumor development in multiple tissue types. However, expression of p53 was inconsistent in corticotroph pituitary adenomas [75,76].

Another cell cycle regulator is pituitary tumor transforming gene 1 (Pttg1), a securin, that controls the separation of chromatids [77,78]. Overexpression of Pttg1 in transgenic mice leads to pituitary hyperplasia and adenoma formation [79]. In human adenoma samples, overexpression of Pttg1 was found in multiple tumor types, including GH-secreting adenomas, non-functioning adenomas and in one corticotroph adenoma [80]. Filipella et al found that PTTG expression was prognostic for recurrence of disease in pituitary adenomas in human patients. In this study, 18 ACTH-secreting adenomas were included [81]. The expression of Pttg1 in dogs has not been investigated yet.

Developmental pathways

Pituitary tumor growth seems to be promoted by hormones that modulate normal pituitary hormonal activity and by growth factors implicated in normal pituitary development [82]. Several growth factors and signaling pathways have been implicated in the pituitary tumorigenic cascade [54]. For example, in non-functioning pituitary adenomas, it was shown that the developmental Wnt and Notch pathways were activated [70]. Although Tpit expression is restricted to corticotroph cells in both normal pituitaries and cortiotroph adenomas, no tumor-specific mutations could be found in canine corticotroph adenoma tissue [83]. Leukemia inhibitory factor (LIF) is important in corticotroph cell lineage differentiation and secretion of ACTH in the adult pituitary gland. Transgenic mice expressing LIF exhibit corticotroph hyperplasia and clinical characteristics of CD [84], but no differential expression of LIF was found in canine pituitary adenomas and normal pituitary tissue [85].

Receptor modulation

Corticotroph pituitary adenomas show overexpression of receptors for CRH and vasopressin [63,66,82,86]. This could lead to overstimulation of the corticotroph cells and contribute to excessive hormone production. Hanson et al showed that nuclear immunoreactivity for LIF receptor in non-tumorous cells of the anterior pituitary may indicate presence of a corticotroph adenoma [85]. Although corticotroph adenomas are usually more resistant to glucocorticoid feedback from the adrenals than normal corticotroph cells, mutations in the glucocorticoid receptors are uncommon [87]. Teshima et al did not find differences in expression of glucocorticoid receptors between corticotroph adenoma tissue and normal pituitary tissue in dogs [88]. Around 60% of pituitary tumors express epidermal growth factor receptors (EGFR) [74]. EGF is a pituitary cell growth factor. In vitro inhibition of EGFR with gefitinib in human, canine and mouse corticotroph pituitary cells leads to decreased tumor cell proliferation and induced apoptosis [89]. EGFR could be a therapeutic target for pituitary-directed therapies. Other possible therapeutic targets include dopamine and somatostatin receptors [59]. The majority of human corticotroph adenomas express somatostatin receptor subtypes (mainly sst5) and dopamine receptor subtype 2 (D2) [58,90], but the expression of these receptors in canine corticotroph pituitary adenomas was much lower [59].

Stem cells in the pituitary gland

In the rat pituitary gland, approximately 30% of cells arise from mitosis of already differentiated cells, whereas the others are produced from undifferentiated cells or possible stem cells [82]. There is increasing interest in the identification of the stem cell population in the pituitary gland from a developmental point of view. Furthermore, stem cells have been implicated to be related to pituitary tumorigenesis [54,91] and are being further explored as a possible anti-cancer therapeutic approach.

The main characteristics of stem cells are ability for self-renewal and capacity to differentiate into multiple lineages. Therefore, stem cells are suggested to play a role in the homeostatic adaptations of the adult pituitary gland, such as the rapid specific cell-type expansion in response to pregnancy or lactation [92-98].

In the past, various cell types have been suggested to act as stem cells in the adult pituitary, such as folliculo-stellate cells, marginal cells, side population (SP) cells or cells expressing Nestin or Sex determining region Y-box 2 (Sox2). Most of these studies have been executed in murine models and all proposed stem cell populations possess several characteristics, such as their location along the Rathke's cleft, their ability to form spheres in culture and to differentiate into the five hormonal cell lineages [95,97-100]. A summary of stem cell characteristics of the different cell types described is given in Table 1.

Table 1. Summary of stem cell characteristics of the different cell types described in this chapter.

Characteristic	Cell types		
	Side Population	**Sox2 Expressing**	**Nestin Expressing**
Localization along Rathke's cleft	Murine pituitary, *Chen et al 2009*	Murine pituitary, *Fauquier et al 2008*	Rat pituitary, *Krylyshkina et al 2005*
Stem cell marker expression	Murine pituitary, *Chen et al 2005/2009*	Murine pituitary, *Fauquier et al 2008, Chen et al 2009*	Murine pituitary, *Gleiberman et al 2008* , Rat pituitary, *Krylyshkina et al 2005*
Sphere formation	Murine pituitary, *Chen et al 2005/2009*	Murine pituitary, *Fauquier et al 2008*	
Differentiation into different hormonal lineages	Murine pituitary, *Chen et al 2009*	Murine pituitary, *Fauquier et al 2008, Chen et al 2009*	Murine pituitary, *Gleiberman et al 2008*

Side Population cells

SP isolation by fluorescence activated cell sorting (FACS) has proven successful to identify and isolate stem and progenitor cells from multiple tissue types [101-104]. This SP phenotype is mediated by membrane efflux pumps of the ATP-binding cassette (ABC)

transporter family; the activity of these pumps is inhibited by ABC-inhibitors such as verapamil, resulting in the disappearance of the SP streak from the FACS plot [93,101,104]. The SP was first isolated in mouse bone marrow and was enriched with hematopoietic stem cells [102]. Since then, SP cells have been shown in numerous other normal tissues, cell lines, and tumors [101,104]. A stem-cell enriched SP was also shown in the murine pituitary gland [93,105]. The pituitary SP represents 1-5% of total cells in the anterior pituitary. It is mainly localized along the Rathke's cleft and has been shown to express many stem cell and progenitor cell markers. Initially, it was shown that in the murine pituitary gland, SP cells expressed the stem cell marker Stem cell antigen 1 (Sca1) [93]. Notably, further dissection of the murine SP revealed that the sub-population not expressing high levels of Sca1 appeared to have the capacity to generate spheres and to differentiate into multiple hormone lineages [105,106]. Hence, further dissection of the heterozygous SP with concomitant functional characterization is needed in order to identify which sub-population is the pituitary progenitor cell population [97,105,106].

Sox2 expressing cells

Sox2 is a member of the SOXB1 group; SOXB1 proteins are widely expressed in the developing neural system. SOXB1 proteins are expressed in pluripotent or undifferentiated cells and their expression is down-regulated when the cells are committed to their cell fate [107]. Together with other transcription factors it maintains the pluripotent capacity of embryonic stem cells [108]. Sox2 is a crucial transcription factor necessary for the function of multiple stem cell populations, especially in the developing central nervous system [97,107]. Sox2 positive cells are also found in craniopharyngiomas, a tumor type arising from the embryonic Rathke's pouch [109].

Sox2 positive cells reside within Rathke's pouch during organogenesis, thereafter Sox2 expression is down-regulated. However, a residual level of Sox2 positivity persists in the adult pituitary [110]. These cells show several stem cell characteristics, such as sphere formation in culture and ability to differentiate into all different hormonal lineages [105,110]. Recent studies showed that Sox2 positive cells are highly upregulated/increased in numbers during pituitary regeneration after partial destruction of pituitary PRL cells, suggesting a role for Sox2 positive cells as stem cells [111,112]. However, also the Sox2 positive cell population is still heterozygous, and will need further analysis to identify stem cells within this population [106]. Within the population of Sox2 expressing pituitary cells, those that are Sox2+/Sox9- have been proposed to be the multipotent stem cells [110].

Nestin expressing cells

Nestin is proposed as a stem cell marker for multiple tissues, including neural tissue [106], and pituitary tissue. During pituitary organogenesis, progenitor cells localize around

Rathke's cleft. These marginal cells have been proposed as a niche of pituitary stem cells in the adult pituitary and were shown to express Nestin [97,100,113]. Also, Nestin-expressing cells can differentiate into all hormonal lineages of the pituitary gland, one of the hallmarks of pituitary stem cells [100,114]. However, on the basis that it is not present early on in the developing pituitary and that it appeared in Sox-2 spheres in vitro (Fauquier 2008), it is believed that it may play a role in the stem cell development rather than be a specific a marker for pituitary stem cells [115].

Cancer stem cells within canine pituitary adenomas

Stem and progenitor cells are also thought to play a role in pituitary tumorigenesis [54,91]. Similar to normal tissues, tumors display different cell populations. One population is thought to be a small group of tumor-initiating cells, the so-called cancer stem cells (CSCs) [116]. These cells are not necessarily transformed normal stem cells, but derive their name from their ability to drive the growth and development of a tumor [100]. It is thought that CSCs are both derived from normal stem cells and from differentiated cells [117]. Stem cell fate is under strict control and deregulation of this controlled system is thought to play a role in tumorigenesis [100]. Furthermore, the recognition of putative stem cells in the adult pituitary gland also raised the suspicion of a role for CSCs in pituitary adenomas. It is important to further investigate the role of stem cells in pituitary adenomas as they could represent a new target for anti-cancer treatment.

So far, publications on the role of stem cells in pituitary tumors are rare, especially in dogs. However, several recent studies indicate an important role for stem and progenitor cells in pituitary tumorigenesis, mostly in mouse models. Stem cell markers have been identified in Rb +/- mice, the most widely used mouse model to study pituitary adenomas [114]. Also, sphere forming cells have been isolated from human pituitary adenomas [118] and SP isolation and characterization has been performed on human pituitary adenomas [96]. In a mouse model, Hosoyama et al showed that a Pax7-expressing cell lineage gave rise to pituitary tumors, also suggesting a role for stem or progenitor cells [119]. From the current knowledge it is likely that canine corticotroph adenomas contain stem cells that drive tumor growth and development. Further studies are needed to identify and characterize this cell population and to develop specific cell targeting therapeutic strategies as a new way of treating canine Cushing's disease.

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3 / Long-term follow-up of dogs with pituitary-dependent hypercortisolism treated with transsphenoidal hypophysectomy

Manuscript in preparation

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Abstract

Transsphenoidal hypophysectomy is one of the tools in the comprehensive management of dogs with pituitary-dependent hypercortisolism (PDH). This study describes the long-term results in a cohort of 306 dogs with PDH treated with hypophysectomy over a 20 year period. Four weeks after surgery, 91% of dogs were alive and remission was confirmed in 92% of these dogs. The median survival time was 781 days (range 0 – 3808 days) with estimated 1 and 5 year survival rates of 86% and 64%, respectively. Median disease free interval was 951 days (range 31 – 3808 days) with estimated 1 and 5 year disease free fractions of 89% and 57%, respectively. Over time, 27% of dogs developed recurrence of hypercortisolism after a median period of 555 days (range 44 – 1688 days). Dogs with recurrence had a significantly higher pituitary height/brain area (P/B) ratio ($P < 0.001$) and pre-operative basal urinary corticoid-to-creatinine ratio (UCCR) ($P = 0.009$) than dogs without recurrence. The survival time ($P = 0.003$) and disease free interval ($P = 0.002$) of dogs with an enlarged pituitary was significantly shorter than in dogs with a non-enlarged pituitary. Pituitary size, reflected by the pituitary height/brain area (P/B) ratio, significantly increased over time ($P < 0.001$), indicating that patients with smaller tumors tended to be treated medically first instead of surgically. Although larger tumors have a less favorable prognosis, the recurrence rate found in this study is comparable to previous reports indicating an improved outcome in larger tumors over time.

The results of this study confirm that transsphenoidal hypophysectomy is an effective treatment for PDH in dogs, with a good long-term outcome. Survival time and disease free fractions are correlated negatively with pituitary size, making the P/B ratio an important pre-operative prognosticator.

Keywords: pituitary surgery, Cushing's disease, Kaplan-Meier, survival, canine

Introduction

Pituitary-dependent hypercortisolism (PDH), also called Cushing's disease, is an endocrine disorder in dogs, caused by an adrenocorticotropic hormone (ACTH)-secreting tumor in the pituitary gland. The estimated incidence is 1 or 2 cases per 1000 dogs per year [1]. Clinical signs are caused by glucocorticoid excess, due to chronic stimulation of the adrenals by the high ACTH levels, and may include polyuria, polydipsia, polyphagia, muscle atrophy, central fat accumulation, alopecia and lethargy [2].

Treatment can be directed at the adrenal gland, where elimination of glucocorticoid excess is achieved via destruction of adrenocortical tissue with the adrenocorticolytic drug o,p'-DDD or with inhibition of glucocorticoid synthesis with trilostane [2]. Treatment at the pituitary level mainly consists of surgical removal of the pituitary gland via transsphenoidal hypophysectomy or radiation therapy [3,4]. Pituitary surgery is also considered the treatment of choice in human patients with Cushing's disease [5].

In 1993, transsphenoidal hypophysectomy was reintroduced as treatment in dogs with PDH at Utrecht University, the Netherlands. Short-term (<3 years) results in 52 dogs and long-term results in 150 and 181 dogs were reported previously [6-8]. The last study of Hanson et al. (2007) reported an initial remission rate of 86% and a recurrence rate of hypercortisolism of 23%. The estimated 1-year and 3-year survival fractions were 84% and 68%, respectively [7]. Main prognostic factors for long-term remission in dogs were pituitary size, thickness of the sphenoid bone, plasma α-melanocyte stimulating hormone (α-MSH) concentration, and urinary cortisol excretion before surgery [8]. Recently, Fracassi et al. reported the long-term follow-up and prognostic factors in dogs with PDH that are treated with trilostane. Median follow-up in 85 patients was 852 days with a 1-year survival of 70% and a 3-year survival of 29%. Age and serum phosphate concentrations were found to be prognosticators for survival time [9].

The aims of this study are to describe the long-term results of transsphenoidal hypophysectomy in a large cohort of dogs with PDH and to redefine the influence of pituitary size on successful surgical outcome. Therefore, we determined survival times, disease-free fractions and recurrence rates and related these parameters to the pituitary size in 306 dogs with PDH treated with transsphenoidal hypophysectomy over a 20 year period.

Materials and Methods

Animals

Three hundred and six dogs with PDH, referred to the Department of Clinical Sciences of Companion Animals, Utrecht University, the Netherlands, over a 20-year period (1993-2013) underwent transsphenoidal hypophysectomy as the primary treatment. Dogs of 76 different breeds and crossbred dogs were included (Table 1). There were 164 male dogs and 142 female dogs. Median age at time of surgery was 8.9 y (range 2.7 – 14.4 y), and median body weight was 17.2 kg (range 3.7 – 61.0 kg).

Table 1. Breeds of 306 dogs with pituitary-dependent hypercortisolism included in the study.

Breed	No
Dachshund	23
Maltese	16
Beagle	14
Labrador Retriever	14
Miniature Poodle	13
Jack Russell Terrier	10
Boxer	9
Yorkshire Terrier	9
Golden Retriever	8
English Cocker Spaniel	6
German Pointer	6
Hovawart	5
Stabyhoun	5
Bouvier des Flandres	4
Cavelier King Charles Spaniel	4
French Bulldog	4
German Shepherd	4
Poodle	4
Shih Tzu	4
Crossbred	66
Other Breeds	78

Diagnosis of PDH was based on clinical signs, results of routine blood examination and measurements of the urinary corticoid-to-creatinine ratio (UCCR) in combination with an oral high-dose dexamethasone suppression test, as described previously [10]. Basal UCCR ranged from 1.7 to 2799.4 x 10^{-6} and median suppression was 80%. In 36 dogs (12%) with less than 50% suppression of the UCCR in the third sample, dexamethasone-resistant PDH was demonstrated by measurements of the plasma ACTH concentration and further supported by visualization of the adrenals by ultrasonography and pituitary imaging with computed tomography (CT) or magnetic resonance imaging (MRI) [11,12]. In dogs with PDH, enlarged pituitaries were distinguished from non-enlarged pituitaries by their P/B ratio (pituitary height (in mm)/brain area (in mm^2)), as described previously [13]. Enlarged pituitaries have a P/B ratio greater than 0.31 and non-enlarged pituitaries have a ratio equal to or less than 0.31.

Surgery and follow-up

All dogs were treated with transsphenoidal hypophysectomy by the same neurosurgeon and peri-operative treatment was according to previously described protocols [6]. Dogs were treated with life-long hormone substitution therapy with cortisone acetate (Cortisoni acetas; Genfarma, Maarssen, the Netherlands) in a dosage of 0.25 mg/kg every 12 hours, and thyroxine (L-thyroxine; Aesculaap, Boxtel, the Netherlands) in a dosage of 15 µg/kg every 12 hours. Desmopressin (Minrin, Ferring, Hoofddorp, the Netherlands), one drop in the conjunctival sac every 8 hours, was administered for 2 weeks routinely and continued if polyuria due to central diabetes insipidus persisted.

The first revisit for clinical examination of the dogs was usually scheduled at 8 weeks after surgery at the endocrinology outpatient clinics. After surgery, UCCR measurements were performed at 2 weeks, 8 weeks, 6 months and thereafter once a year, or more frequently in dogs suspected of recurrence of hypercortisolism. All morning urine samples for UCCR measurements were collected at home by the owner when the dog was 24 hours free of cortisone acetate medication, and samples were sent to our laboratory by mail or brought in on control visits. Follow-up reports were obtained from the routine follow-up examinations in the clinic, or during telephone conversations with the owner and/or referring veterinarian. Postoperative mortality was diagnosed as death within 4 weeks after surgery. Remission was defined as UCCR < 10 x 10^{-6} and resolution of clinical signs of hypercortisolism. Residual disease was defined as early postoperative (<8 weeks) UCCR > 10 x 10^{-6}. Recurrence was defined as UCCR ≥ 10 x 10^{-6} and return of clinical signs of hypercortisolism after initial remission.

Statistical analysis

All analyses were made with IBM® SPSS®Statistics for Windows, Version 20.0. (Armonk, NY). Survival and disease free fractions were analyzed by the Kaplan-Meier estimation procedure. Dogs that died of unrelated causes or were alive at last follow-up were counted as censored cases. Differences between Kaplan-Meier curves were compared with the Log Rank test. Comparisons between dogs with and without recurrence were done with Mann Whitney-U test for non-parametric data. P/B ratios over time were analyzed with the Kruskal-Wallis test. Significance was set at $P < 0.05$.

Results

Postoperative mortality

Twenty seven dogs (8.8%) died within 4 weeks after surgery (Figure 1). The median P/B ratio of this subgroup was 0.54 (range 0.21 to 1.40). The P/B ratio of dogs that died within 4 weeks after surgery was significantly higher than the P/B ratio of dogs that were alive at 4 weeks ($P = 0.004$, Figure 2). Twelve of these cases have already been described [7]. Of the others dogs, two dogs were euthanized shortly after surgery because they did not recover from anesthesia. Three dogs stayed in a comatose state for several days and were euthanized on the intensive care unit within 5 days. One dog was euthanized after three days because of severe hypernatremia. Five dogs were euthanized or died spontaneously because of postoperative dyspnea; in one of these dogs a pulmonary thromboembolism was found during postmortem pathology. One dog died at home, four days after surgery for unknown reasons. Two dogs were euthanized because of persistent vomiting after release from the hospital. One dog developed a septic peritonitis due to an intestinal foreign body two weeks after surgery and was euthanized during emergency surgery.

Long-term survival

Two hundred seventy nine dogs were alive at 4 weeks after surgery (Figure 1). Of these dogs, six were lost to follow-up and therefore no disease status was available. Remission was confirmed in 257 dogs (84% of the total patient group, 92% of dogs alive at 4 weeks) with a UCCR $< 10 \times 10^{-6}$ and resolution of clinical signs of hypercortisolism at 8 weeks after surgery. Residual disease (UCCR $> 10 \times 10^{-6}$ at 8 weeks) was present in 16 dogs, i.e. 5.7% of dogs alive at 4 weeks. Seven of these dogs were euthanized within five months for reasons associated with hypercortisolism. One dog was treated with bilateral adrenalectomy, and survived for 34 months. One dog was diagnosed with ectopic ACTH production which was described previously by our group [14]. Four dogs were treated with o,p'-DDD (Lysodren, Bristol-Meyers Squibb, Utrecht, the Netherlands), follow-up time of these dogs was 160 days, 237 days, 594 days and 783 days at last follow-up. Two

dogs were treated with trilostane (Vetoryl, Dechra, Shrewsbury, UK) with a follow-up of 594 and 1967 days. One dog with residual disease had no clinical signs and was euthanized after 783 days because of a liver tumor.

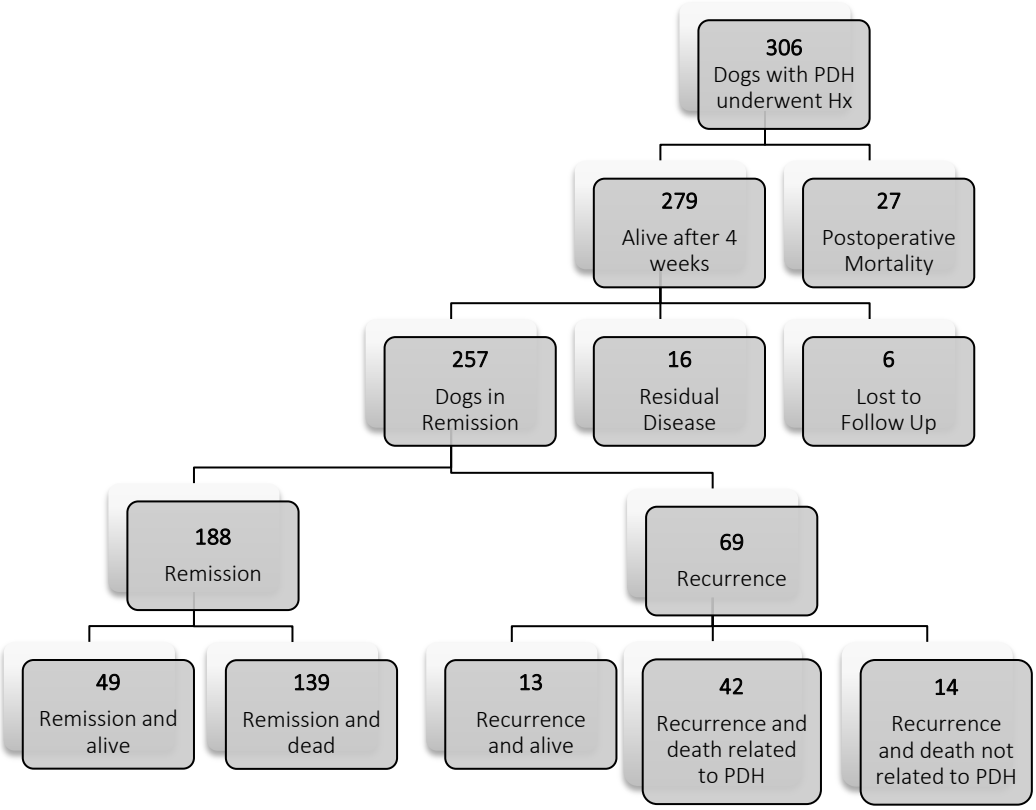

Figure 1. Flowchart of 306 dogs with pituitary-dependent hypercortisolism (PDH) that underwent transsphenoidal hypophysectomy (Hx) in the period 1993 – 2013.

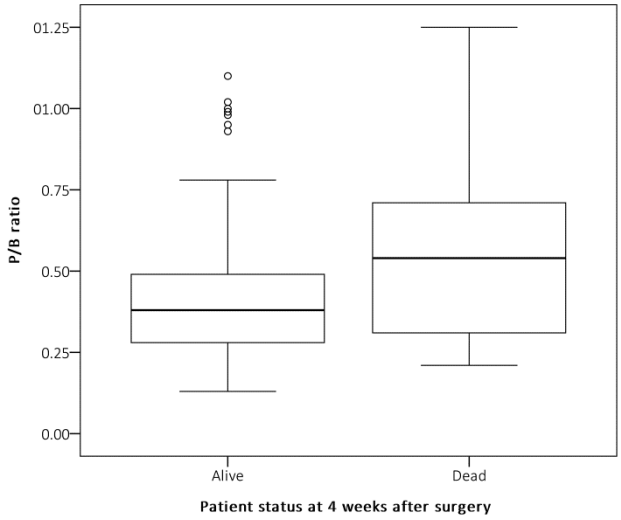

Figure 2. Boxplots displaying the pituitary height/brain area (P/B) ratio of dogs alive and dead at 4 weeks after transsphenoidal hypophysectomy as treatment for pituitary-dependent hypercortisolism. The P/B ratio of dogs that died within 4 weeks after surgery is significantly higher (P = 0.004). ◦ indicate outliers.

The median survival time of the 300 dogs from which follow-up date were available was 781 days (range 0 – 3808 days) (Figure 3A). Estimated 1-year survival rate was 86% (SE 2%), estimated 2-year survival rate was 79% (SE 3%), estimated 3-year survival rate was 74% (SE 3%), estimated 4-year survival rate was 72% (SE 3%) and estimated 5-year survival rate was 64% (SE 4%).

The disease-free interval was analyzed for 257 dogs with confirmed remission of hypercortisolism after surgery. Median disease-free interval was 951 days (range 31 – 3808 days) (Figure 4A). Estimated 1-year, 2 year, 3-year, 4-year and 5-year disease-free fractions were 89% (SE 2%), 79% (SE 3%), 74% (SE 3%), 64% (SE 4%) and 57% (SE 4%), respectively.

Recurrences

In 188 of 257 dogs (73%), hypercortisolism was in remission at the time of analysis. Of these dogs, 139 had died or were euthanized because of non-Cushing-related causes, after a median period of 935 days after hypophysectomy (range 31 – 3808 days). Over time, 69 of 257 (27%) dogs had recurrence of hypercortisolism after a median period of 555 days (range 44 – 1688 days).

At last follow-up 13 of these dogs were alive, 42 had died or were euthanized because of recurrent signs of hypercortisolism and 14 died of non-related causes (Figure 1). Dogs with recurrence of hypercortisolism had a significantly higher P/B ratio ($P < 0.001$) and pre-operative basal UCCR ($P = 0.009$) than dogs without recurrence (Figure 5).

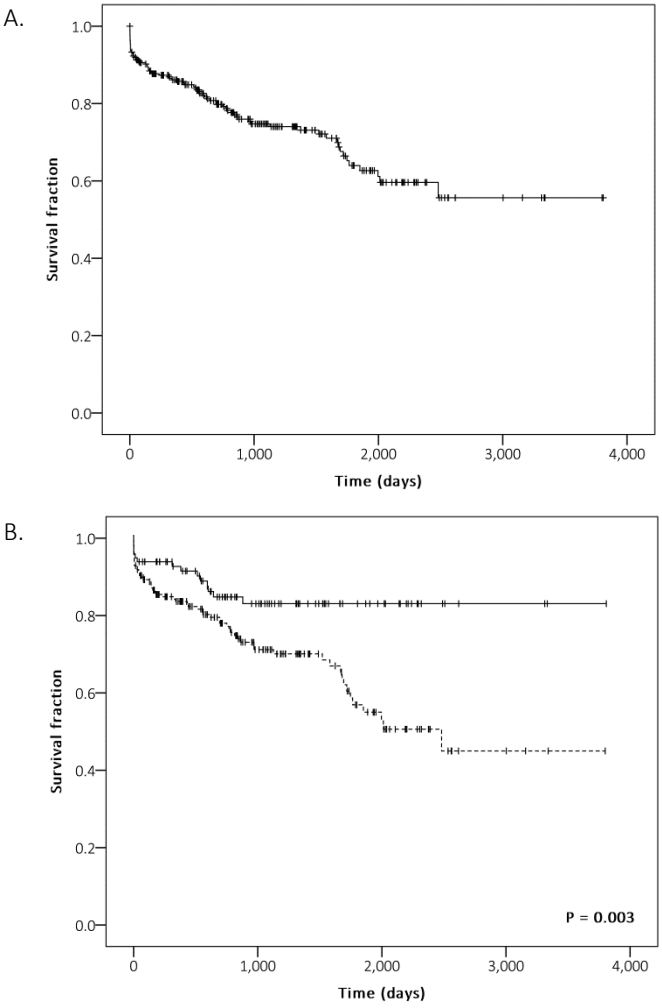

Figure 3. A) Survival curve of 300 dogs after transsphenoidal hypophysectomy as treatment for pituitary-dependent hypercortisolism. Censored cases (i.e. dogs that died from unrelated causes or were still alive at last follow-up) are represented with vertical bars. B) Survival curves for dogs with an enlarged pituitary (P/B > 0.31; dotted line) and non-enlarged pituitary (P/B ≤ 0.31; continuous line). P = 0.003.

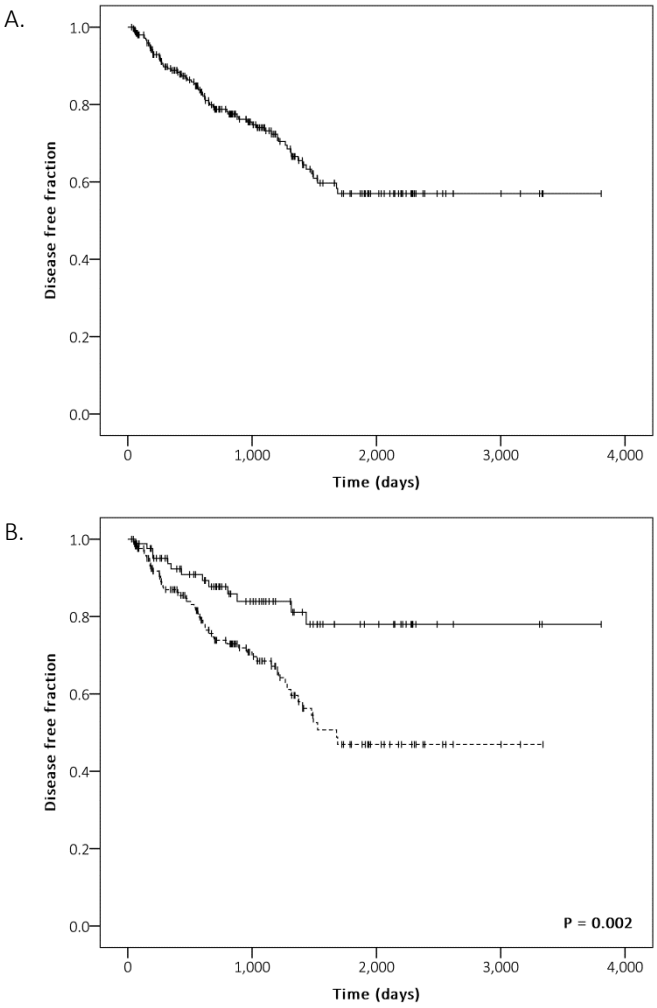

Figure 4. A) Disease-free fraction curve of 257 dogs with post-operative remission after transsphenoidal hypophysectomy as treatment for pituitary-dependent hypercortisolism. Censored cases (i.e. dogs that died from unrelated causes or were still alive at last follow-up) are represented with vertical bars. B) Disease-free fraction curves for dogs with an enlarged pituitary (P/B > 0.31; dotted line) and non-enlarged (P/B ≤ 0.31; continuous line). P = 0.002.

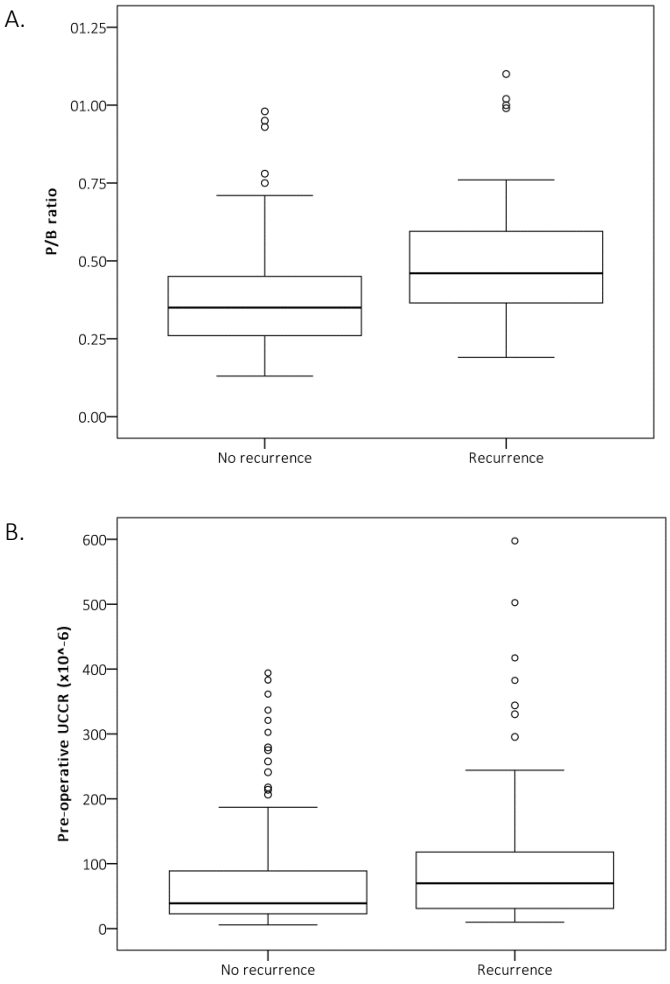

Figure 5. A) Boxplots of the P/B ratio of 257 dogs with and without recurrence after transsphenoidal hypophysectomy as treatment for pituitary-dependent hypercortisolism (P < 0.001); B) Boxplots of the pre-operative urinary corticoid-to-creatinine ratio (UCCR) of dogs with and without recurrence (P = 0.009). ◦ indicate outliers.

Pituitary size

In this study, median P/B ratio was 0.39 (range 0.13 – 1.40). The pituitary was enlarged in 201 dogs (median 0.47, range 0.32 – 1.4) and non-enlarged in 100 dogs (median 0.25, range 0.13 - 0.31). The P/B ratio was unavailable in 5 dogs. No significant correlation was found between the P/B ratio and pre-operative UCCR. The survival time (P = 0.003) and disease-free interval (P = 0.002) of dogs with an enlarged pituitary was significantly shorter than those in dogs with a non-enlarged pituitary (Figures 3B, 4B).

Pituitary size, reflected by the P/B ratio, significantly increased over the time period 1993-2013 with the median P/B ratio of the dogs operated between 1993-1997 (n=74) of 0.32, in 1997-2003 (n = 75) of 0.34, in 2003-2007 (n = 75) of 0.4, and in 2007-2013 (n = 77) of 0.5 (Figure 6, P < 0.001).

The postoperative remission rate increased over time (P = 0.08). It was 79% in the period 1993-1997, 87% in 1997-2003, 87% in 2003-2007 and 83% in 2007-2013. The recurrence rate in these four time periods did not differ significantly (P = 0.14). It was 19% in the period 1993-1997, 37% in 1997-2003, 24% in 2003-2007 and 26% in 2007-2013.

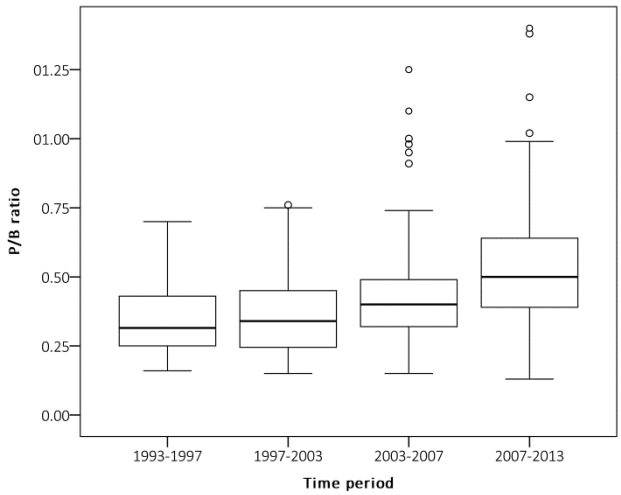

Figure 6. Boxplots of P/B ratios of 301 dogs that underwent transsphenoidal hypophysectomy as treatment for pituitary-dependent hypercortisolism. The dogs were divided in four quartiles of 74 to 77 dogs based on the time of surgery over a 20-year period (1993-2013). A significant increase in P/B ratio was found (P < 0.001). ◦ indicate outliers.

Discussion

Since our first report in 1998, on the results of transsphenoidal hypophysectomy in 52 dogs [6], the number of operated dogs has steadily increased to 306 dogs. With a median follow-up of >2 years and the longest follow-up of more than 10 years after surgery, the present study shows long-term results and confirms that transsphenoidal hypophysectomy is an effective treatment of dogs with PDH.

In the present study, postoperative mortality was 9% and postoperative remission rate was 84%, which was the same as reported previously [7]. The remission rate increased over time, from 79% in 1993-1997 to 83% in 2007-2013. Remission rates in human pituitary surgery for Cushing's disease varies from 42-93%, depending on the definition used [5]. In dogs with Cushing's disease the most common medical treatment is with trilostane, a competitive inhibitor of the 3β-hydroxysteroid dehydrogenase/isomerase system which is essential for the synthesis of cortisol [2]. Reported remission rates of clinical signs in dogs after trilostane are 70-86% [15,16]. Large case series such as the present study are not available for medical treatment, but reported follow-up is around 900 days [9,15,16]. Since medical treatment is not directed at the pituitary tumor itself, expansion of the pituitary lesion may lead to neurological signs by the mass effect. For these patients hypophysectomy or radiotherapy are the only options left for treatment.

The estimated survival and disease-free intervals slightly improved compared to our previous study. Recurrence rate in the present study was 27%, a small increase from the previously reported of 25% [7]. Possibly, this is caused by the increase in pituitary size of the patients that were referred for hypophysectomy over the past decade. With the introduction of trilostane treatment, dogs with a non-enlarged pituitary tend to be treated medically first, whereas the dogs with an enlarged pituitary are frequently referred for surgery. The median P/B ratio in the present study significantly increased over time indicating a tendency to refer primarily dogs with an enlarged pituitary for surgery. Both survival time and disease-free interval were shown to be significantly lower in dogs with a larger pituitary gland. Also, dogs that died within 4 weeks of the surgery had a significantly higher P/B ratio. This was also shown in human studies, where pituitary size was the main prognosticator [17,18]. However, the overall long-term results were not very different from our results reported in previous series and the recurrence rate was not significantly different between the four time periods. Apparently, the negative effect of larger pituitary size on survival and disease-free fractions was compensated by the increase of experience of the pituitary surgeon in dealing with larger pituitary tumors and improvement of perioperative care.

We also found that the pre-operative UCCR of dogs that developed recurrence was higher than in dogs without recurrence. This was also shown in humans, where high urinary cortisol concentrations were predictive for recurrence [19]. It is speculated that ACTH and thus cortisol secretion is related to pituitary size and therefore to a less favorable prognosis [13]. In the present study, no significant correlation between P/B ratio (reflecting pituitary size) and pre-operative UCCR was found.

It is concluded that transsphenoidal hypophysectomy is an effective treatment for PDH in dogs with a good long-term outcome. Survival time and disease-free fractions decrease with increasing pituitary size, which indicates that the P/B ratio is an important pre-operative prognosticator. In general, a 9-year-old dog that is diagnosed with Cushing's disease and which is treated by transsphenoidal surgery, has an average life expectancy of another 2 to 3 years when remission is confirmed within 8 weeks after surgery.

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4/ The prognostic value of peri-operative profiles of ACTH and cortisol for recurrence after transsphenoidal hypophysectomy in dogs with corticotroph adenomas

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Abstract

Background: Transsphenoidal hypophysectomy is an effective treatment for dogs with pituitary-dependent hypercortisolism (PDH). However, long-term recurrence of hypercortisolism is a well-recognized problem, indicating the need for reliable prognostic indicators.

Objectives: To evaluate the prognostic value of peri-operative plasma ACTH and cortisol concentrations for identifying recurrence of hypercortisolism after transsphenoidal hypophysectomy.

Animals: 112 dogs with PDH that underwent transsphenoidal hypophysectomy met the inclusion criteria of the study.

Methods: Hormone concentrations were measured pre-operatively and 1 to 5 h after surgery. Both absolute hormone concentrations and postoperative concentrations normalized to preoperative concentrations were included in analyses. The prognostic value of hormone concentrations was studied with Cox's proportional hazard analysis.

Results: Median follow-up and disease-free period were 1096 days and 896 days, respectively. 28% of patients had recurrence, with a median disease-free period of 588 days. Both absolute and normalized postoperative cortisol concentrations were significantly higher in dogs with recurrence than in dogs without recurrence. High ACTH 5 h after surgery, high cortisol 1 h and 4 h after surgery, high normalized ACTH 3 h after surgery, high normalized cortisol 4 h after surgery and the random slope of cortisol were associated with a shorter disease-free period.

Conclusions and clinical importance: Individual peri-operative hormone curves provide valuable information about the risk of recurrence after hypophesectomy. However, because no single cut-off point could be identified, combination with other variables, such as the pituitary height/brain area (P/B) ratio, is still needed to obtain a good estimate of the risk for recurrence of hypercortisolism after hypophysectomy.

***Keywords*:** hypercortisolism, Cushing´s disease, canine, survival analysis

Introduction

Pituitary-dependent hypercortisolism (PDH) is a common endocrinopathy in dogs, with an estimated incidence of 1 or 2 in 1000 dogs/year [1]. It is caused by an adrenocorticotropic hormone (ACTH) secreting pituitary adenoma. Pituitary carcinomas are rare [2]. Clinical signs are caused by hypercortisolism and include polyuria, polydipsia, polyphagia, panting, heat intolerance, enlargement of the abdomen caused by centripetal fat storage, atrophy of the skin, alopecia, muscle weakness and lethargy [3]. Pituitary-dependent hypercortisolism in dogs has many similarities to Cushing's disease (CD) in humans; which has a much lower incidence, affecting only 1 to 2 persons per million [4,5].

Pituitary surgery is the treatment of choice for CD in human patients, in whom preferably an adenomectomy is performed [6]. In dogs, transsphenoidal hypophysectomy has been shown to be an effective treatment with PDH [7,8]. Despite high initial remission rates, long-term recurrence is a well-recognized problem in both humans [9-13] and dogs [7,14], with reported recurrence rates of 8.5% in humans and 25% in dogs [7,12]. Therefore, much effort has been expended to identify reliable predictors for completeness of tumor removal and recurrence, either pre-operatively or postoperatively.

In both humans and dogs, recurrence is more common in patients with an enlarged pituitary gland [10,15]. For postoperative evaluation of pituitary surgery in humans, different protocols have been used including intraoperative and postoperative measurement of plasma cortisol [9,16-20] and ACTH [21-23] concentrations, as well as stimulation tests with corticotropin-releasing hormone (CRH) [6,24,25] and desmopressin [25-27]. Overall, postoperative basal hormone concentrations in humans are the most important predictor for recurrence [12], but a test that is predictive for the individual patient remains to be developed [28].

For postoperative evaluation of transsphenoidal hypophysectomy in dogs, the urinary corticoid-to-creatinine ratio (UCCR) has been used to define residual disease, remission, and recurrence. Urinary corticoid-to-creatinine ratio values in the upper half of the reference range 6-8 weeks after surgery are associated with a higher frequency of recurrence at long-term follow-up, whereas low UCCR values predict long-term remission [15]. The UCCR is an indirect reflection of ACTH production by the pituitary gland, therefore the plasma ACTH concentration may give more direct information about the completeness of hypophysectomy and risk for recurrence. Because of the short plasma half-life of ACTH (approximately 20 minutes in both humans and dogs), the plasma ACTH concentration should decrease within a few hours after hypophysectomy [29,30]. This makes direct postoperative evaluation of ACTH concentrations very interesting and it is hypothesized that very low ACTH concentrations within a few hours after

hypophysectomy will be associated with long-term remission. Indeed, in humans it was shown that patients with increased plasma ACTH concentrations developed recurrence, even though their postoperative serum cortisol concentrations were low [22]. In dogs with PDH, a combined stimulation test, including CRH and 3 other hypophysiotrophic hormones, performed at 8 weeks after hypophysectomy, in combination with administration of three other hypophysiotropic releasing hormones, failed to identify dogs in which the disease would recur [31], but plasma ACTH and cortisol concentrations collected immediately after hypophysectomy have not been evaluated yet.

The aim of the present study was to evaluate the prognostic value of peri-operative plasma ACTH and cortisol concentrations for recurrence of hypercortisolism after transsphenoidal hypophysectomy in dogs with PDH.

Materials & Methods

Animals

Between 2001-2013, 204 dogs with PDH, referred to the Department of Clinical Sciences of Companion Animals, Utrecht University, the Netherlands, underwent transsphenoidal hypophysectomy as primary treatment for PDH. All dogs were operated by the same veterinary neurosurgeon (BM). Medical records of these dogs were evaluated retrospectively, and dogs were included in this study when the post-operative follow-up period was ≥ 1 year and post-operative remission of PDH was confirmed within 8 weeks after surgery. Thirty-eight dogs were excluded because follow-up was < 1 year, 25 dogs were excluded because remission was not confirmed within 8 weeks after surgery and 29 dogs were excluded because the hormone measurements were not performed. In total, 112 dogs met the inclusion criteria, consisting of 10 Labrador Retrievers, 9 Maltese dogs, 8 Beagles, 8 Dachshunds, 5 Boxers, 4 Jack Russell Terriers, 3 Yorkshire Terriers, 3 Stabyhouns, 3 German Pointers, 2 Bearded Collies, 2 Cavalier King Charles Spaniels, 2 Hovawarts, 2 Scottish Terriers, 1 dog each of 33 other breeds, and 18 crossbred dogs. There were 53 male dogs (21 castrated) and 59 female dogs (45 spayed). Age at time of surgery ranged from 3.7 to 14 years (median, 8.5 years). Body weight ranged from 3.8 to 61 kg (median, 19.5 kg).

Diagnosis

The diagnosis of hypercortisolism was based on clinical signs and increased UCCRs (reference range, 0.3-8.3×10^{-6} [32]) in 2 consecutive morning urine samples collected at home [33]. After collection of the second urine sample, 3 doses of 0.1 mg dexamethasone per kg body weight were administered PO at 6-8 h intervals and the next morning a third urine sample was collected. When the UCCR in the third sample was < 50% of the mean of that of the first 2 samples, the dog was categorized as being responsive to dexamethasone suppression, and PDH was diagnosed [33]. The median UCCR of the 112 dogs included in this study was 55x10^{-6} (range, 6 - 652×10^{-6}), and the median suppression after dexamethasone was 80% (range, -162 - 99%). In 36 cases there was < 50% suppression of the UCCR in the third sample, and in these dogs dexamethasone-resistant PDH was demonstrated by measurement of plasma ACTH concentration. The diagnosis of PDH was further supported by visualization of the adrenal glands by ultrasonography and pituitary gland imaging using computed tomography (CT) or magnetic resonance imaging (MRI) [34,35]. In dogs with PDH, enlarged pituitary glands were distinguished from non-enlarged pituitary glands by their pituitary height/brain area (P/B) ratio [36]. A microadenoma in a non-enlarged pituitary gland can be visualized indirectly by the displacement of the 'pituitary flush' on dynamic CT imaging [35]. For the dogs included in this study, the median P/B ratio was 0.43 (range, 0.13 to 1.1). There were 24 dogs with non-enlarged pituitary glands (P/B ≤0.31) and 88 dogs with enlarged pituitary glands (P/B >0.31).

Treatment and follow-up

Transsphenoidal hypophysectomy was performed according to a microsurgical technique described previously [37]. After removal of the pituitary gland, treatment was started with 1 drop of 0.01% desmopressin[a] and continued every 8 h in the conjunctival sac. Intravenous administration of 1 mg hydrocortisone[b] per kg body weight was started 5 h after removal of the pituitary gland and continued every 6 h until the dog resumed eating and drinking. Postoperative treatment followed the same protocol as described previously [38]. The dogs were kept on life-long substitution therapy with cortisone acetate[c] at a dosage of 0.25 mg/kg PO q12h, and thyroxine[d] at a dosage of 15 µg/kg PO q12h. Desmopressin was administered routinely for 2 weeks and continued if polyuria due to central diabetes insipidus persisted [38]. The dogs were re-examined after 8 weeks. After surgery, UCCR was measured (in duplicate) at 2 weeks, 8 weeks, 6 months and thereafter once a year, or more frequently in dogs with suspected recurrence. All morning urine samples for UCCR measurements were collected at home by the owner 24 h after cortisone acetate treatment. Follow-up reports were obtained from the routine follow-up examinations in the clinic, or during telephone conversations with the owner, referring veterinarian, or both. Remission was defined as UCCR < 10x10^{-6} and resolution of clinical

signs of hypercortisolism. Recurrence was defined as UCCR ≥ $10x10^{-6}$ and reappearance of clinical signs of hypercortisolism after initial remission.

Blood sampling and analysis

The protocol was approved by the Ethical Committee of the Faculty of Veterinary Medicine (Utrecht University, Utrecht, The Netherlands). Two basal blood samples were collected on the day before surgery before 2008 and within 2 hours before the surgery after 2008. The average of the 2 values was included in this study as the pre-operative value. After hypophysectomy, the first blood sample was collected when the dog was still on the operating table, approximately 1 h after extraction of the pituitary gland. The remaining samples were collected at 2, 3, 4, and 5 h after hypophysectomy when the dog was recovering in the intensive care unit. Desmopressin was started when the dog left the operating table. Hydrocortisone medication was started after the 5 h sample was collected. Blood samples were collected in pre-chilled EDTA tubes and kept on ice, and centrifuged for 10-12 minutes at 4°C. The plasma was stored at -20 °C until analyzed. Plasma ACTH concentration was measured by a commercially available 2-site immunoradiometric assay[e,f]. Plasma cortisol concentration was measured by a solid phase 125I radioimmunoassay[g] [39].

Data analysis

Calculations were performed with SPSS[h] and R Studio[i]. For all postoperative time points, normalized plasma ACTH and cortisol concentrations were calculated by dividing the absolute plasma concentration by the pre-operative value. These normalized plasma ACTH and cortisol concentrations also were used for analysis. Also, for each dog the area under the curve (AUC), random slope (RS) and random intercept (RI) of the ACTH and cortisol curves were calculated. Normality was assessed with the Kolmogorov-Smirnov Test and, non-parametric tests were used accordingly. Correlations were calculated using the Spearman rho test. Differences between dogs with and without recurrence were evaluated with Mann-Whitney U tests. Longitudinal comparisons were made using the Wilcoxon rank sum test. Bonferoni corrections for multiple comparisons were applied.

The disease-free period was calculated for all dogs and was defined as the interval between the date of surgery and the date on which the dog was last known to be in remission, or the date of recurrence. Dogs that had died from nonrelated causes and dogs that were still alive and in remission at the time of follow-up were counted as censored cases. To assess the prognostic value of the different factors, variables were first analyzed with univariate Cox's proportional-hazard analysis. Variables with a P-value <0.05 were then entered in a multivariate Cox's proportional-hazard analysis (Reverse Stepwise [Conditional LR]). For variables significant in the multivariate analysis, cut-off values to create the binary variables 'low' vs. 'high' were calculated based on a receiver operating

curve (ROC) [40]. Disease-free analysis was performed using Kaplan-Meier curves and the Log Rank test was used to assess significance between 'low' and 'high' values. Significance was set at $P < 0.05$.

Results

Follow-up data

Median follow-up for the 112 patients included in this study was 1096 days (range 373–3799 days). At last follow-up, 35 dogs were alive. Median disease-free period was 896 days (range 80–3340 days). Of the 112 patients, 31 (28%) had recurrence of hypercortisolism, whereas 81 dogs were still in remission at last follow up (alive or dead due to nonrelated cause). Median disease-free period for the dogs with recurrence was 588 days (range 80–1688 days).

Basal hormone concentrations and clinical variables

Correlation was calculated between pre-operative plasma ACTH and cortisol concentrations and clinical variables (P/B ratio, pre-operative UCCR, survival time and disease-free period). A significant correlation was only found between pre-operative plasma ACTH concentration and P/B ratio ($\rho=0.28$, $P=0.003$) and between pre-operative plasma cortisol concentration and pre-operative UCCR ($\rho=0.31$, $P=0.003$).

Hormone profiles after hypophysectomy

For each dog, plasma ACTH and cortisol concentrations were plotted as an individual curve (Figure 1) and compared with follow-up information. A significant correlation was found between absolute plasma ACTH and cortisol concentrations at 1 to 5 h after surgery, normalized plasma ACTH and cortisol concentrations at 1 to 5 h after surgery, AUC of ACTH and cortisol, and RI of ACTH and cortisol. For each time point of ACTH and cortisol measurement, plasma concentrations, normalized plasma concentrations and curve characteristics were compared between the group of dogs with recurrence and the group of dogs without recurrence.

Plasma cortisol concentrations at 1 to 5 h after surgery were significantly higher in dogs with recurrence than those in dogs without recurrence (Figure 2). Also, significant differences were found in normalized plasma cortisol concentrations at 1 to 5 h after surgery and for RS and RI of cortisol. When comparing the lowest postoperative result, dogs with recurrence had a significantly higher plasma cortisol concentration ($P = 0.008$), and plasma ACTH concentration tended to be higher in these dogs ($P = 0.07$). Data also were analyzed longitudinally, both for dogs with recurrence and dogs without recurrence.

Plasma ACTH and cortisol concentrations decreased significantly over time after surgery in both groups (Figure 2).

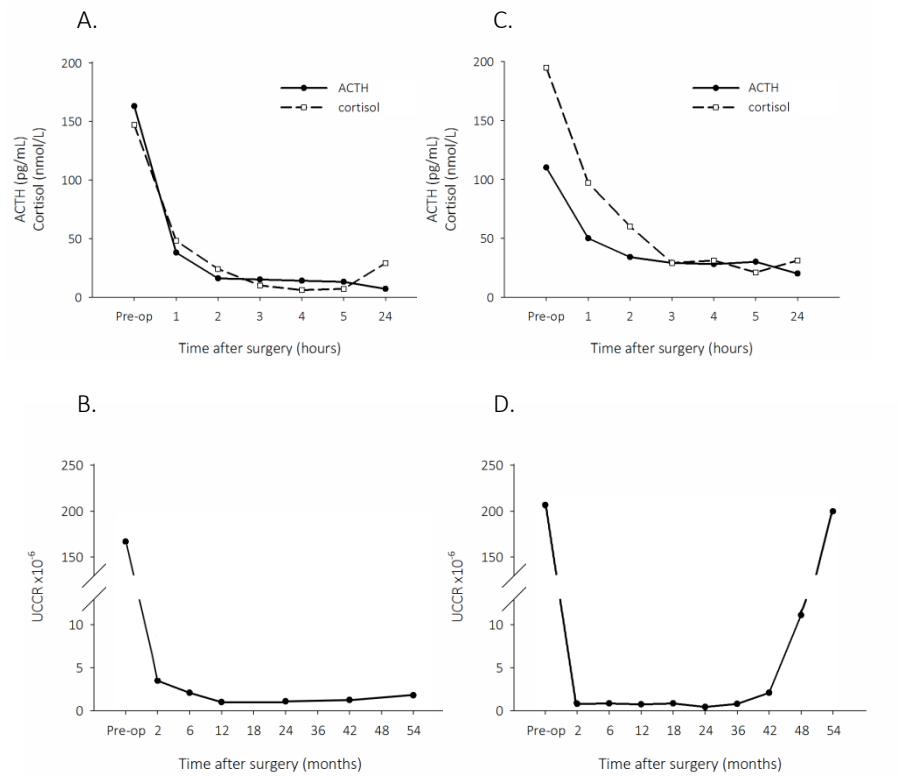
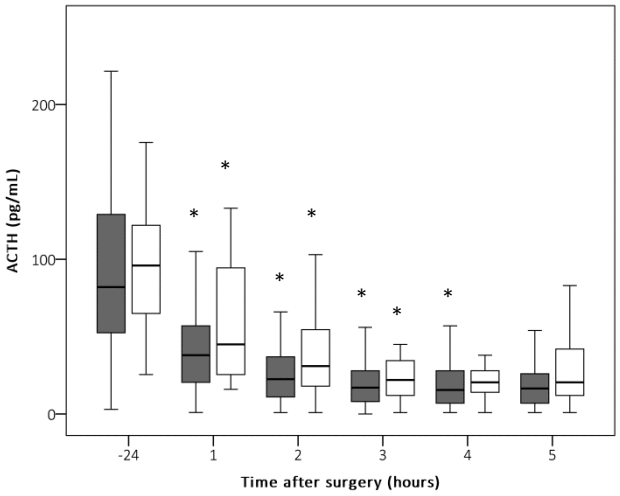


Figure 1. Typical example of peri-operative individual profiles of plasma ACTH and cortisol concentrations in dogs that underwent hypophysectomy for treatment of PDH. Panels A and C show the plasma concentrations of ACTH and cortisol from 24h (Pre-op) before to 5h after surgery. Panels B and D show the UCCRs of the same dogs over time. The dog in panel A and B remained in remission for 54 months, and the plasma ACTH and cortisol concentrations decreased within 5 hours after hypophysectomy to values close to zero. The dog in panel C and D had a recurrence of hypercortisolism at 48 months after surgery. In this dog plasma ACTH and cortisol concentrations did not decrease to values close to zero within 5 hours after hypophysectomy.

A.



B.

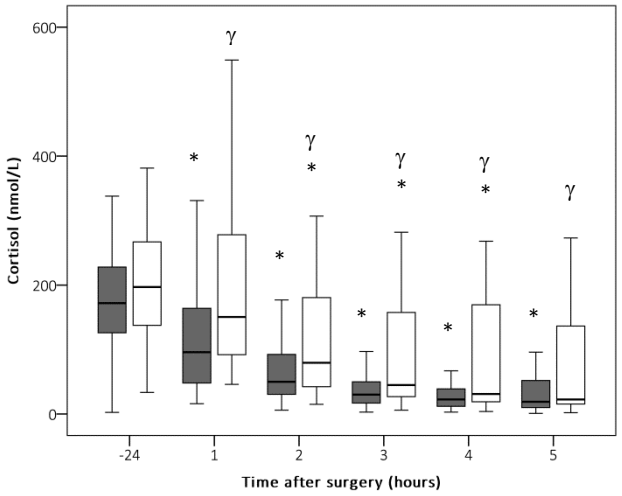


*Figure 2. Boxplots demonstrating the change in plasma ACTH (A) and cortisol (B) concentrations over time in dogs with recurrence (open boxes, n=31) and dogs without recurrence (black boxes, n=81) of hypercortisolism after hypophysectomy. * indicates a significant difference to the previous time point. Y indicates a significant difference between plasma cortisol concentrations in dogs with and without recurrence.*

Disease-free period analysis

Clinical variables (P/B ratio, age, body weight, sex), absolute plasma ACTH and cortisol concentrations, normalized plasma ACTH and cortisol concentrations and curve characteristics (AUC, RI and RS) were entered in the univariate Cox's proportional hazard analysis for disease-free period of the 112 dogs. The variables that were significant ($P < 0.05$, Table 1) were entered in a multivariate Cox's proportional hazard model, grouped in absolute plasma hormone concentrations, normalized plasma hormone concentrations, and curve characteristics. The P/B ratio was excluded from further analysis, fr a better appreciation of the influence of the hormone concentrations in the different models.

Table 1. Significant variables (P<0.05) in the univariate Cox's proportional hazard analysis for disease-free period after transsphenoidal hypophysectomy. Variables with P>0.05 are left out of this table.

Variable	HR	95% CI		P value
P/B value	22.664	4.478	114.712	<0.001
ACTH2	1.008	1.002	1.014	0.007
ACTH3	1.007	1.003	1.010	<0.001
ACTH4	1.016	1.008	1.025	<0.001
ACTH5	1.019	1.004	1.035	0.014
Cort1	1.004	1.002	1.005	<0.001
Cort2	1.004	1.002	1.005	<0.001
Cort3	1.002	1.001	1.004	<0.001
Cort4	1.004	1.003	1.006	<0.001
normACTH2	4.477	1.416	14.152	<0.001
normACTH3	2.241	1.469	3.420	<0.001
normACTH4	4.788	1.770	12.954	0.002
normCort1	1.826	1.318	2.530	<0.001
normCort2	1.888	1.286	2.772	0.001
normCort3	1.498	1.176	1.908	0.001
normCort4	2.067	1.407	3.035	<0.001
RI ACTH	1.011	1.003	1.018	0.005
AUC Cort	1.000	1.000	1.000	<0.001
RI Cort	1.005	1.003	1.008	<0.001
RS Cort	1.171	1.093	1.256	<0.001

normACTH = normalized plasma ACTH concentration to preoperative value. normCort = normalized plasma cortisol concentration to preoperative value, RI = random intercept, RS = random slope, AUC Cort = area under the curve for cortisol, HR = hazard ratio, CI = confidence interval.

High plasma ACTH concentration 5 h after surgery, high plasma cortisol concentrations 1 h and 4 h after surgery, high normalized plasma ACTH concentration 3 h after surgery, high normalized plasma cortisol concentration 4 h after surgery and the RS of cortisol were associated with a shorter disease-free period (Hazard Ratio >1.0, P <0.05) (Table 2). No significant differences were found between Kaplan-Meier survival curves for disease-free periods of these variables (ACTH 5 h after surgery [P = 0.36], cortisol 1 h after surgery [P=0.12], cortisol 4 h after surgery [P=0.07, Figure 3], normalized cortisol 4 h after surgery [P=0.15] and RS of cortisol [P=0.13]). The ROC curve of normalized ACTH 3 h after surgery was not of enough quality to calculate a cut-off value.

Table 2. Grouped variables entered in multivariate Cox's proportional hazard analysis (Reverse LR Conditional) for disease-free period after transsphenoidal hypophysectomy resulted in a final model.

Grouped variables entered	Variables in final model	HR	95% CI		P value
ACTH2, ACTH3, ACTH4, ACTH5, Cort1, Cort2, Cort3, Cort4	ACTH3	1.032	0.999	1.066	0.060
	ACTH4	0.921	0.845	1.005	0.064
	ACTH5	1.055	1.006	1.106	0.026*
	Cort1	1.005	1.000	1.010	0.041*
	Cort2	0.993	0.986	1.001	0.072
	Cort4	1.007	1.002	1.013	0.008*
normACTH2, normACTH3, normACTH4, normCort1, normCort2, normCort3, normCort4	normACTH3	2.082	1.237	3.504	0.006*
	normCort4	1.805	1.182	2.756	0.006*
RI ACTH, RI Cort, RS Cort, AUC Cort	RI ACTH	1.011	0.999	1.022	0.067
	RS Cort	1.150	1.064	1.244	<0.001*

*Significant variables (P<0.05) are marked with *. normACTH = normalized plasma ACTH concentration to preoperative value, normCort = normalized plasma cortisol concentration to preoperative value, RI = random intercept, RS = random slope, AUC Cort = area under the curve for cortisol, HR = hazard ratio, CI = confidence interval.*

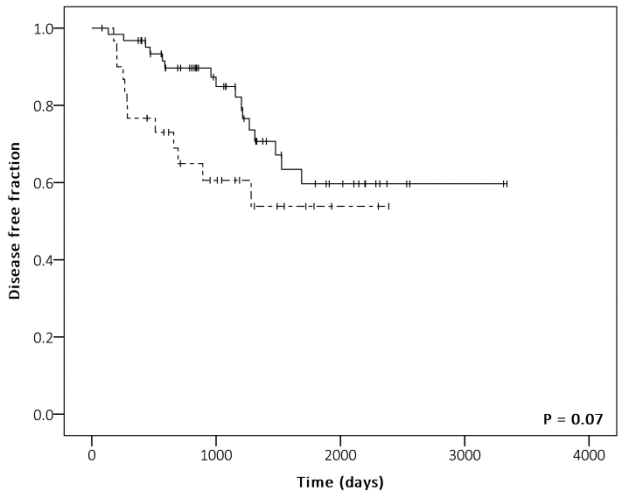


Figure 3. Kaplan-Meier curves comparing the disease-free period in dogs with high (>33 nmol/L), dotted line, n=31) and low (<33 nmol/L), continuous line, n=62) plasma cortisol concentrations at 4h after hypophysectomy (P = 0.07). Censored cases are represented by vertical bars.

Discussion

We studied the prognostic value of peri-operative ACTH and cortisol concentrations in predicting recurrence of hypercortisolism in dogs after hypophysectomy. With multivariate analysis we identified several variables with significant prognostic value (ACTH 5 h after surgery, cortisol 1 h and 4 h after surgery, normalized ACTH 3 h after surgery, normalized cortisol 4 h after surgery, and the RS of cortisol). However, Kaplan-Meier survival analysis showed that single time point measurements were not sufficient to predict a significant difference in disease-free period, as was also shown in a meta-analysis of studies of humans [12]. Therefore, peri-operative hormone concentrations must be combined with other clinical information, such as pituitary size (P/B ratio), to provide reliable information about the prognosis of dogs with PDH.

The long-term recurrence rate in this study was 28% (31 in 112 dogs). The dogs included in this study had a median P/B ratio of 0.43, indicating a high proportion of dogs with enlarged pituitary glands. We previously showed that high P/B ratio is predictive of recurrence [15], and also in this study, the P/B ratio had a high hazard ratio in the Cox's proportional hazard's analysis.

We showed a significant decrease of the plasma ACTH concentrations after surgery in both dogs with and without recurrence. The short half-life of ACTH explains this rapid drop. Plasma ACTH concentrations have been measured intra-operatively in humans [21-23], but are not commonly used as prognostic indications. A recent study of humans did however show the usefulness of the combination of ACTH and cortisol measurements [22]. Plasma cortisol has a longer half-life (in humans approximately 50 minutes [41]), and the decrease in plasma cortisol concentration after hypophysectomy is expected to be less steep than that of ACTH. In humans, plasma and urinary cortisol concentrations are most commonly used as prognostic variables [9,12,16-20]. We showed also that cortisol concentrations decreased significantly in the postoperative period, with the median plasma cortisol concentration being significantly higher in dogs that developed recurrence than in dogs without recurrence at all postoperative time points.

Evaluating individual hormone curves for patients with and without recurrence, we observed a faster decrease in plasma ACTH concentrations for dogs without recurrence, but the mean plasma ACTH concentrations were not different between groups, probably because of large variation, especially in the recurrence group. Very low plasma ACTH concentrations suggest complete removal of the corticotroph adenoma, but do not exclude presence of remaining normal pituitary cells, because these cells have been suppressed by the negative feedback of high cortisol concentrations for a long period. Very low plasma ACTH concentrations within 5 h as an indication of complete hypophysectomy is even more noteworthy because at 1 h after hypophysectomy desmopressin was administered which, besides replacing the vasopressin deficiency, is a well-known stimulant of ACTH secretion [25-27]. Because of this, measurement of high postoperative plasma ACTH concentrations indicates remnant functional corticotroph adenoma tissue, increasing the risk for recurrence of hypercortisolism over time. Indeed, evaluating the lowest postoperative plasma concentrations of ACTH and cortisol for each patient, we showed significantly higher plasma cortisol concentrations in dogs with recurrence.

With multivariate analysis, not only absolute hormone concentrations were included, but also normalized concentrations to the preoperative result and characteristics of the complete hormone curve. The definitive models included several variables with significant prognostic value. The advantage of hourly-repeated measurements is that urve characteristics could be included in the analysis. Kaplan-Meier survival analysis showed that single time point measurements were not sufficient to predict recurrences, as was also shown in a meta-analysis of studies of humans [12], making a combination of different data essential.

Based on a cohort of 112 patients with a follow up of >1 year, a combination of absolute hormone concentrations, normalized hormone concentrations and curve characteristics has a prognostic value. Therefore, use of the hormone curve of a patient, in combination with other clinical variables (such as P/B ratio) is needed in the postoperative evaluation of dogs with PDH in order to provide a reliable estimation of the prognosis. Close follow-up of the patients, over the long-term, also remains crucial.

Footnotes

a Minrin, Ferring, Hoofddorp, the Netherlands

b Solu-cortef, Upjohn, Ede, the Netherlands

c Cortisoni acetas; Genfarma, Maarssen, the Netherlands

d L-thyroxine; Aesculaap, Boxtel, the Netherlands

e Nichols Institute, Wijchen, the Netherlands (until 2007)

f Diasorin S.A./N.V., Brussels, Belgium (from 2007)

g Coat-a-Count® Cortisol, DPC (Siemens healthcare diagnostics), the Hague, the Netherlands

h IBM SPSS®Statistics for Windows, Version 20.0, Armonk, NY, USA

i R Studio, version 0.98, http://www.rstudio.com

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5 / Expression of Ki-67, PCNA, and p27kip1 in canine pituitary corticotroph adenomas

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Abstract

Pituitary-dependent hypercortisolism (PDH), which is caused by adrenocorticotropic hormone (ACTH)-secreting pituitary adenomas, is a common endocrinopathy in dogs. Dogs with non-enlarged pituitaries harboring a microadenoma have a better prognosis than those with enlarged pituitaries. The aim of this study was to investigate the expression of the proliferation markers Ki-67 and proliferating cell nuclear antigen (PCNA) and the cell-cycle inhibitor p27kip1 in corticotroph adenomas in enlarged and nonenlarged pituitaries. The expression of Ki-67, PCNA, and p27kip1 was analyzed by immunohistochemical staining of 17 pituitary adenoma samples harvested during pituitary surgery in dogs with PDH. The labeling index was calculated by counting the number of immunopositive cells per 1,000 cells. The mean (± standard deviation) labeling index for Ki-67 was 8.4%±14.2% for the group with enlarged pituitaries, and 8.8%±5.5% for the group with non-enlarged pituitaries; that for PCNA was 35.5%±12.2% and 37.0%±15.5%; and that for p27kip1 was 29.3%±22.6% and 42.5%±27.9%, respectively. No significant differences in Ki-67, PCNA, and p27kip1 labeling indices were found between enlarged and non-enlarged pituitaries. However, a trend toward significance was observed when comparing the expression of p27kip1 in enlarged pituitaries versus normal pituitary tissue. It is concluded that Ki-67 and PCNA are not useful as proliferative markers for studying the pathobiology of pituitary corticotroph adenomas in dogs.

Keywords: Pituitary-dependent hypercortisolism; Dog; Immunohistochemistry

Introduction

Adrenocorticotropic hormone (ACTH)-secreting pituitary adenomas cause pituitary-dependent hypercortisolism (PDH or Cushing's disease), which is a common endocrinopathy in dogs [1]. In humans, adenomas larger than 10mm are called macroadenomas, and adenomas smaller than 10mm are called microadenomas. In dogs with clinical signs caused by hormone-producing pituitary neoplasms, a distinction is made between adenomas in enlarged and those in non-enlarged pituitaries, measured as the ratio between the height of the pituitary gland and the area of the brain (P/B) [2]. Enlarged pituitaries have a P/B ratio > 0.31 and non-enlarged pituitaries have a ratio ≤ 0.31. Pituitary adenomas in dogs behave differently than those found in humans. In 10%-30% of dogs with PDH, the pituitary is enlarged, but invasive growth and metastasis are rare [3]. Hanson et al. [1,4] showed that the outcome of pituitary surgery is associated with tumor size, with the prognosis being worse for large tumors.

Although relatively little is known about the pathobiology of canine pituitary adenomas, the pathobiology of human pituitary adenomas has been studied extensively. Prognostic factors for other types of tumor, such as mutations in ras and p53, are not relevant in pituitary adenomas [5], and so other markers are needed that can help predict disease outcome and facilitate the choice of postoperative therapy. In humans, Ki-67 and proliferating cell nuclear antigen (PCNA), markers of cell proliferation, are widely used to predict pituitary adenoma behavior and surgical outcome. Since the Ki-67 antigen is expressed in G1, S, G2, and M phases of the cell cycle, but not in G0, it distinguishes between proliferating cells and quiescent cells [6]. In humans, the Ki-67 labeling index (LI) of corticotroph macroadenomas and microadenomas is significantly different [7], as is the mean Ki-67 LI of recurrent and nonrecurrent adenomas after surgical excision. An elevated Ki-67 LI is associated with tumor invasiveness [8-13].

Proliferating cell nuclear antigen accumulates in the nucleus during the S phase of the cell cycle. Therefore, it seems to have a greater specificity than Ki-67 [14]. However, Atkin et al. [15] showed that PCNA seemed to overestimate proliferation, whereas Ki-67 did not. In humans, PCNA was found to be higher in recurrent pituitary adenomas than in nonrecurrent adenomas [16,17].

The cell-cycle inhibitor p27kip1 inhibits cyclin cyclin–dependent kinase complexes, regulating cell cycle transition from the G1 to the S phase [18,19]. Dysregulation of G1–S transition is a common feature of human tumors, which suggests that p27kip1 is involved in tumor growth [20]. Indeed, p27kip1 knockout mice develop pituitary tumors that originate from corticotroph cells in the intermediate lobe [21-23], and a decrease in p27kip1 expression is considered a negative prognostic factor in breast and colon cancer

[22,23]. The p27kip1 LI is lower in pituitary tumors than in normal pituitary tissue and is correlated with tumor recurrence [18,23,24].

Although PDH is a common endocrinopathy in dogs, the expression of Ki-67, PCNA, and p27kip1 has not been investigated in canine pituitary adenomas. The aim of the present study was to investigate the expression of Ki-67, PCNA, and p27kip in canine pituitary corticotroph adenomas and compare the expression of these markers in enlarged and non-enlarged pituitaries.

Materials and methods

Animals

The present study was approved by the Ethics Committee on Animal Experimentation of the Faculty of Veterinary Medicine, Utrecht University, the Netherlands. Seventeen dogs with PDH that had undergone transsphenoidal hypophysectomy [25] were included in this study. The dogs (6 females and 11 males) included 1 Beagle, 1 Bearded Collie, 1 Bernese Mountain Dog, 1 Cavalier King Charles Spaniel, 1 Chesapeake Bay Retriever, 1 English Cocker Spaniel, 1 Irish Terrier, 1 Maltese, 1 Miniature Poodle, 1 Collie, 3 Yorkshire Terriers, and 4 crossbred dogs. The median age at the time of surgery was 8.5 y (range: 5.6 to 12.4 y) (Table 1). The diagnosis of hypercortisolism was based on an elevated ($\geq 10\times10^{-6}$) urinary corticoid-to-creatinine ratio (UCCR) measured in 2 consecutive morning urine samples collected by the owner. Immediately after collection of the second urine sample, the animals received 3 doses of 0.1 mg dexamethasone/kg PO at 8-h intervals. The next morning, a third urine sample was collected. If the UCCR of the third sample was less than 50% of the mean of the first 2 samples, the dog was considered to be responsive to dexamethasone suppression, and PDH was diagnosed. The median UCCR of the 17 dogs included in this study was 57×10−6 (range: 21-234×$10^{-6)}$, and in all cases, the UCCR was suppressed more than 50% by dexamethasone (median 90%, range: 61% to 99%) (Table 1). The diagnosis of PDH was confirmed by measurement of plasma ACTH concentrations (Table 1) and further supported by visualization of the adrenals by ultrasonography and pituitary imaging [1,4,26].

The dogs were grouped according to the P/B ratio determined on magnetic resonance imaging (MRI) or computed tomography (CT) scans before surgery (Table 1). Nine dogs had enlarged pituitaries (median P/B 0.69, range: 0.34 to 1.10), and 8 dogs had nonenlarged pituitaries (median P/B 0.23, range: 0.18 to 0.31) (Table 1).

Table 1. Clinical characteristics of the patients included in this study.

No	Group	Breed	Age (y)	Gender	Weight (Kg)	UCCR[a] ($x10^{-6}$)	Dexamethasone Suppression[b]	ACTH[c] (pg/mL)	α-MSH[d] (pg/mL)	Cortisol[e] (nmol/L)	Pituitary Size[f] (height x width x length)	P/B ratio[g]
1	NE	Miniature Poodle	11.2	F*	9.9	41.0	90.0%	35.0	4.5	189.0	3.3 x 5 x 5	0.23
2	NE	Yorkshire Terrier	5.8	M	6.8	234.0	91.5%	25.5	16.5	372.0	4.1 x 4.6 x 5	0.31
3	NE	Yorkshire Terrier	9.2	F*	6.0	82.0	92.8%	158.0	190.0	326.0	5 x 5 x 5	0.27
4	NE	Irish Terrier	8.8	M*	24.7	29.5	93.6%	122.5	50.0	201.0	5 x 7 x 6	0.30
5	NE	Yorkshire Terrier	7.2	M*	8.0	59.5	93.9%	62.0	4.0	280.5	5 x 5 x 4	0.27
6	NE	Scottish Collie	9.8	M	29.1	20.5	89.3%	221.5	24.0	186.5	3.6 x 4.6 x 5.6	0.22
7	NE	Cavelier King Charles Spaniel	6.4	M	13.5	41.0	99.3%	79.5	6.5	61.0	3.2 x 4.6 x 4.1	0.18
8	NE	Crossbred	8.5	M*	19.9	31.0	77.4%	18.5	8.0	138.5	5 x 6 x 6	0.24
9	E	Crossbred	10.1	M*	25.2	57.0	94.4%	73.5	8.0	265.0	11.5 x 10.6 x 10	0.70
10	E	English Cocker Spaniel	5.8	F	16.7	96.1	69.4%	107.0	14.5	338.0	14.2 x 17.4 x 17	1.10
11	E	Bearded Collie	9.8	F*	24.9	22.5	92.9%	73.5	16.5	131.0	6.4 x 8.1 x 6.3	0.34
12	E	Maltese	10.4	F*	3.7	213.5	67.2%	633.0	366.0	184.5	13.8 x 14.4 x 15.3	0.91
13	E	Crossbred	7.3	M	23.0	NA	NA	71.0	618.0	198.0	10.4 x 11 x 11.3	0.65
14	E	Bernese Mountain Dog	6.7	F	48.0	100.0	61.0%	190.0	4.5	319.0	11.7 x 13 x 11.7	0.69
15	E	Crossbred	12.4	M*	14.2	27.7	93.1%	147.5	23.0	161.5	7.4 x 8.6 x 7.3	0.44
16	E	Beagle	5.6	M*	16.3	217.5	80.2%	94.5	12.5	277.5	6.5 x 9.3 x 10	0.38
17	E	Chesapeake Bay Retriever	7.1	M	41.5	65.0	66.2%	72.0	4.0	157.5	11.9 x 13.9 x 16.5	0.70

*Abbreviations: *, castrated; α-MSH, α-melanocyte-stimulating hormone; ACTH, adrenocorticotropic hormone; E, enlarged pituitary; F, female; M, male; NA, not available; NE, non-enlarged pituitary; UCCR, urinary corticoid-to-creatinine ratio. a Preoperative urinary-to-creatinine ratio (reference < 10×10^{-6}); values are the mean of 2 morning urine samples with a 1-d interval. b Preoperative degree of UCCR suppression after high-dose dexamethasone. c Preoperative plasma ACTH (reference 5-85 pg/mL); values are the mean of 2 samples with an interval of 10-15 min. d Preoperative plasma α-MSH (reference <36 pg/mL); values are the mean of 2 samples with an interval of 10-15 min. e Preoperative plasma cortisol (reference 11-136 nmol/L); values are the mean of 2 samples with an interval of 10-15 min. f Pituitary size in mm, as measured on preoperative helical computed tomography. g P/B$\times10^{-2}$ mm^{-1}, ratio of the pituitary size and the brain area. P/B ≤ indicates a non-enlarged pituitary; P/B > 0.31 indicates an enlarged pituitary.*

Histology and immunohistochemistry

Specimens of pituitary tissue removed during surgery were fixed in 4% methanol-formaldehyde, embedded in paraffin, and consecutive sections were used for histology (hematoxylin and eosin [H&E] staining), hormone immunohistochemistry, and staining for Ki-67, PCNA, and p27kip1. The diagnosis of pituitary neoplasia was recognized on H&E staining by its typical adenomatous organization and characteristic basophilic-eosinophilic-chromophobic staining pattern in comparison with normal pituitary tissue. The diagnosis of corticotroph adenoma was confirmed by immunostaining for ACTH, α-melanocyte-stimulating hormone (α-MSH), and growth hormone (GH).

Immunohistochemistry was performed using antibodies in an indirect immunoperoxidase staining procedure, using the avidin-biotin–based technique (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA). Sections (4 µm) were mounted on poly-L-lysine-coated slides (ACTH, GH) or Silan-coated slides (α-MSH). Slides were routinely deparaffinized and rehydrated. Endogenous peroxidase activity was blocked by incubating the slides with 1% H_2O_2 for 30 min. After the slides were rinsed with phosphate-buffered saline with 0.1% Tween-20 (PBS-T; Boom, Meppel, the Netherlands) (3×5 min), they were pre-incubated with normal horse serum in PBS (1:10, ACTH) or normal goat serum in PBS (1:10, α-MSH and GH) for 15 min. Subsequently, the primary antibody was applied (ACTH, 1:100 monoclonal antibody against ACTH, clone 2F6, Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, the Netherlands; α-MSH, 1:400 polyclonal rabbit antibodies to synthetic α-MSH, MZ111; Biomol International, Exeter, UK; GH, 1:5,000 rabbit anti-human antibody STH, N1561, Dako, Glostrop, Denmark) and incubated overnight at 4 °C. After incubation, slides were rinsed in PBS-T (3×5 min) and incubated with biotinylated horse anti-mouse IgG 1:125 in PBS (ACTH) (Vector Laboratories) or biotinylated goat anti-rabbit (mainly) IgG 1:250 in PBS (α-MSH and GH) (Dako) for 30 min. Then the sections were rinsed in PBS-T (3×5 min) and incubated with avidin-biotin complex, freshly prepared according to the manufacturer's instructions, for 30 min. After the slides were rinsed with PBS (3×5 min), immunoreactive CYP1A was visualized using 0.3% H_2O_2 and 0.5% 3,3-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO, USA) diluted in 0.05 mol/L Tris/HCl buffer during a 10-min incubation step. Then, sections were rinsed in distilled water for 10 min, dehydrated, sealed, and covered with coverslips.

For the detection of Ki-67,PCNA, and p27kip1, 4-µm thick sections were mounted on poly-L-lysine-coated slides, deparaffinized, and rehydrated through a series of xylene and graded alcohol to Tris-buffered saline (TBS, 0.01M Tris [hydroxymethyl]-aminomethan, Merck, Darmstadt, Germany, 0.9% NaCl, pH = 7.6). Antigen retrieval was performed in a microwave oven using the following procedures. Slides used for Ki-67 immunostaining

were microwaved in 800mL citrate buffer (10mM citric acid monohydrate, Merck, pH = 6.0) for 7 min at 850W and 15 min at 450W. Slides used for PCNA immunostaining were microwaved in 800mL MilliQ water for 7 min at 850W and 20 min at 450W. Slides used for p27kip1 staining were microwaved in 800mL citrate buffer for 10 min at 850W. Slides were left to cool down to room temperature for 20 min before washing them 2×5 min in TBS-T (TBS with 0.1% Tween-20; Boom). Endogenous peroxidase activity was blocked by 30-min incubation in a solution of 0.3% H_2O_2 in methanol. Slides were washed for 5 min in TBS-T and treated for 30 min with 10% normal goat serum (Dako) in TBS. Incubation with primary antibodies took place overnight, at 4 °C; the anti-p27Kip1 monoclonal mouse antibody (BD Biosciences, Breda, the Netherlands, Clone 57, Isotype IgG1) was diluted 1:250 in TBS, and the anti-Ki67 monoclonal rabbit antibody (LabVision, Duiven, the Netherlands, Clone SP6, Isotype IgG) was diluted 1:50 in TBS. The anti-PCNA monoclonal mouse anti-rat antibody (Dako, Clone PC10, Isotype IgG2a) was used in a ready-to-use solution. After the slides were washed for 2×5 min in TBS-T, they were incubated for 45 min with secondary antibodies: labeled polymer-HRP Anti Mouse for p27kip1 and PCNA immunostaining and labeled polymer-HRP Anti Rabbit for Ki-67 immunostaining (Dako EnVision Systems). Slides were washed for 2×5 min in TBS, before the detection of staining with DAB substrate (Vector Laboratories). After being washed for 5 min in MilliQ water, the slides were counterstained for 10s with hematoxylin and eosin (HE) and washed for 10 min in running tap water. Slides were dehydrated with a series of graded alcohol and xylene and mounted with Vectamount (Vector Laboratories).

Ki-67, PCNA, and p27kip1 antibodies were tested for specificity against canine tissue according to the manufacturer's product information. Positive immunostaining controls were obtained by using canine gut tissue (for Ki-67 and PCNA) and normal canine pituitary tissue (for p27kip1). Normal anterior lobe pituitary tissue was obtained from dogs that were euthanized in other experiments approved by the Ethics Committee on Animal Experimentation of the Faculty of Veterinary Medicine, Utrecht University, the Netherlands. The 4 dogs (2 dogs and 2 bitches; median age 6.5 y, range: 1 to 12 y) were a Bouvier des Flandres, a Greyhound, a Labrador Retriever, and a crossbreed. Negative controls were obtained by omitting the primary antibody.

Scoring for Ki-67, PCNA, and p27kip1

Pituitary corticotroph adenoma localization was determined using the H&E slides and hormone immunostaining slides from the same series. On consecutive, identical slides that were immunostained for Ki-67, PCNA, and p27kip1, the positive cells were identified and counted together with an experienced veterinary pathologist (GG) who is routinely diagnosing canine endocrine disorders. Slides were photographed using an Olympus BX41 microscope (Olympus, Center Valley, PA, USA) connected to a Color View III camera (Soft

Imaging Systems, Münster, Germany). On average, 1,000 cells/slide were counted at 200×magnification, using the touch count method of the AnalySIS Software package (Version 3.2, Released: 2002, Soft Imaging Systems, Münster, Germany). Labeling indices of Ki-67, PCNA, and p27kip1 were obtained by dividing the number of immunopositive cells by the total number of cells.

Statistical analysis

All calculations were performed with R Software, version 2.6.1 (Released: November 2007, Free Software Foundation, Boston, MA, USA). The Ki-67, PCNA, and p27kip1 LI data were compared between the group with enlarged pituitaries and the group with non-enlarged pituitaries. Also, for Ki-67 and p27kip1, the LI of both groups and the LI of the total group of adenomas were compared to the LI of the control pituitaries. The Student's t-test or the Wilcoxon rank sum test was performed to determine significance between the groups. The correlation was investigated between the Ki-67 LI and the p27kip1 LI and between the Ki-67, PCNA, and p27kip1 LI on the one hand and the P/B ratio on the other hand. A Pearson's product-moment correlation test was used to determine significance. Significance was set at $P < 0.05$.

Results

Histology and immunohistochemistry for ACTH and α-MSH

In all dogs, histological examination of the surgical specimen revealed a corticotroph adenoma staining immunohistochemically positive for ACTH and α-MSH and negative for GH (Table 2). The enlarged pituitaries showed histological signs of malignancy, namely, invasive growth or dedifferentiated, proliferating cells.

Scoring for Ki-67, PCNA and p27kip1

On average, 1,000 cells (range 873-2760 cells) were counted in adenoma samples; Ki-67-, PCNA-, and p27kip1-positive cells stained brown (Figs. 1 and 2). The mean (± SD) LI for Ki-67 was 8.8%±15.5% for the group with non-enlarged pituitaries, and 8.4%±14.2% for the group with enlarged pituitaries (Fig. 3, Table 2).

The mean (± SD) LI for PCNA was 37.0%±15.5% for the group with non-enlarged pituitaries, and 35.5%±12.2% for the group with enlarged pituitaries (Fig. 4, Table 2). The mean (± SD) LI for p27kip1 was 42.5%±27.9% for the group with non-enlarged pituitaries, and 29.3%±22.6% for the group with enlarged pituitaries (Fig. 5, Table 2).

Table 2. Summary of the histopathological diagnosis, the patients' follow-up, and the labeling indices for Ki-67, PCNA, and p27kip1.

No	Group	Recur.[a]	Survival (mo)	Histopathological diagnosis[b]	Immunohistochemistry[c]	Labelling Index[d]		
						Ki-67	PCNA	p27kip1
1	NE	no	3	microadenoma	NA	1.9	42.9	51.7
2	NE	yes	54.6*	microadenoma	ACTH +, αMSH +, GH -	0.0	29.1	35.3
3	NE	no	14.8	microadenoma	ACTH +, αMSH +, GH -	2.0	38.8	73.0
4	NE	no	7.6	adenoma	ACTH -, αMSH +, GH -	6.6	8.6	10.8
5	NE	no	47.8*	adenoma	ACTH +, αMSH +, GH -	0.0	47.4	39.3
6	NE	no	44.5	infiltrative adenoma	ACTH +, αMSH +, GH -	1.0	55.1	84.6
7	NE	no	32.0*	multinodular adenoma	ACTH +, αMSH +, GH -	13.2	49.7	43.0
8	NE	no	28.8*	adenoma	ACTH +/-, αMSH +/-, GH -	45.4	24.2	2.7
9	E	no	43.9	adenoma	NA	17.1	27.3	4.5
10	E	yes	27.1	adenoma	ACTH +/-, αMSH +/-, GH -	43.3	47.3	77.4
11	E	no	36.6*	adenoma	ACTH +, αMSH +, GH -	2.1	42.2	20.5
12	E	no	0.1	adenoma	ACTH +, αMSH NA, GH -	0.1	47.4	27.6
13	E	no	27.0*	adenoma	ACTH +/-, αMSH +/-, GH -	3.6	12.6	9.1
14	E	no	22.0*	adenoma	ACTH +, αMSH +/-, GH -	0.0	22.5	16.8
15	E	no	15.1*	infiltrative adenoma	ACTH +, αMSH +, GH -	0.0	42.8	24.6
16	E	no	10.7*	adenoma	ACTH +/-, αMSH +, GH -	1.7	34.3	51.2
17	E	no	9.3*	adenoma	ACTH +/-, αMSH +, GH -	8.0	43.0	31.6
18	HC			normal pituitary	NA	1.3	NA	64.4
19	HC			normal pituitary	NA	1.6	NA	41.6
20	HC			normal pituitary	NA	6.8	NA	55.5
21	HC			normal pituitary	NA	6.5	NA	60.5

*Abbreviations: *, alive; +, marked immunoreactivity, +/-, weak immunoreactivity, -, no immunoreactivity; E, enlarged pituitary; HC, healthy control; NA, not available; NE, non-enlarged pituitary. a. Recurrence of the disease after initial remission. b,c Diagnosis as stated by a veterinary pathologist based on hematoxylin and eosin staining and immunohistochemistry for ACTH, α-MSH, and GH. d Labeling index: number of positive cells/total number of cells counted.*

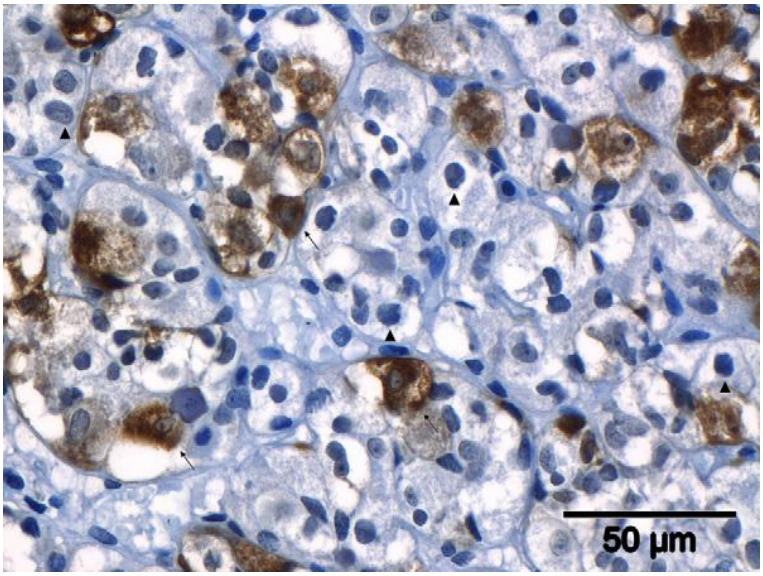

Figure 1. Ki-67 immunohistochemistry. Adenoma cells, part of a microadenoma present in a non-enlarged pituitary. Positive cells stain brown (arrows), whereas negative cells remain blue (arrowheads).

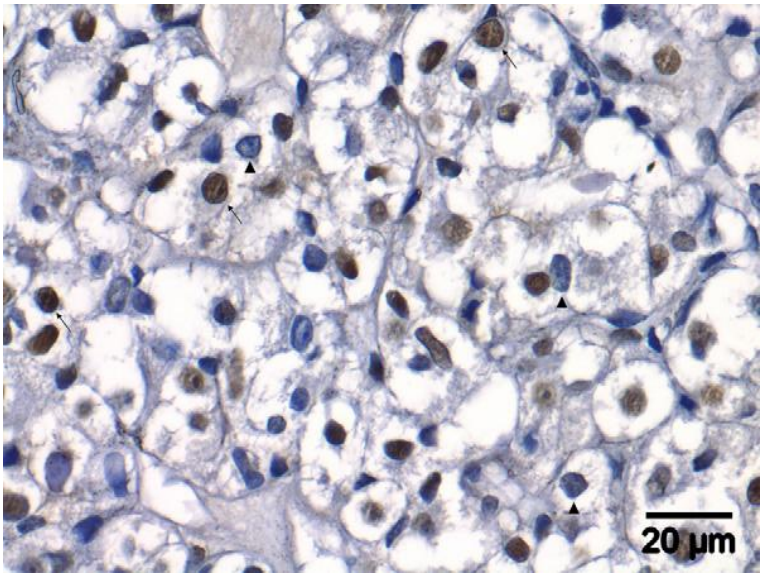

Figure 2. p27kip1 immunohistochemistry. Adenoma cells, part of a microadenoma present in a non-enlarged pituitary. Positive cells stain brown (arrows), whereas negative cells remain blue (arrowheads).

An average of 1,000 cells (range 909 to 1157 cells) was counted in control pituitary samples stained for Ki-67 and p27kip1. The mean LI for Ki-67 was 4.1%±3.0% (Fig. 3, Table 2), and that for p27kip1was 55.5%±9.9% (Fig. 4, Table 2). The Ki-67 LI in enlarged and non-enlarged pituitaries was not significantly different (P = 0.96), nor was that of adenoma samples and control pituitary samples (enlarged vs control: P = 1, non-enlarged vs. control: P = 0.93, all adenomas vs control: P = 0.96). The PCNA LI in enlarged and non-enlarged pituitaries was not significantly different (P = 0.83). The p27kip1 LI in enlarged and non-enlarged pituitaries was also not significantly different (P = 0.30), nor was that of non-enlarged and control pituitary samples (P=0.40); however, the p27kip1 LI of enlarged pituitaries was nearly significantly lower than that in the control pituitary tissue (P = 0.05). The Ki-67 and p27kip1 LIs were not correlated (P = 0.87), and neither were the Ki-67, PCNA, and p27kip1 LIs correlated with the P/B ratio (Ki-67 LI and P/B, P=0.36; PCNA LI and P/B, P = 0.67; p27kip1 LI and P/B, P = 0.87).

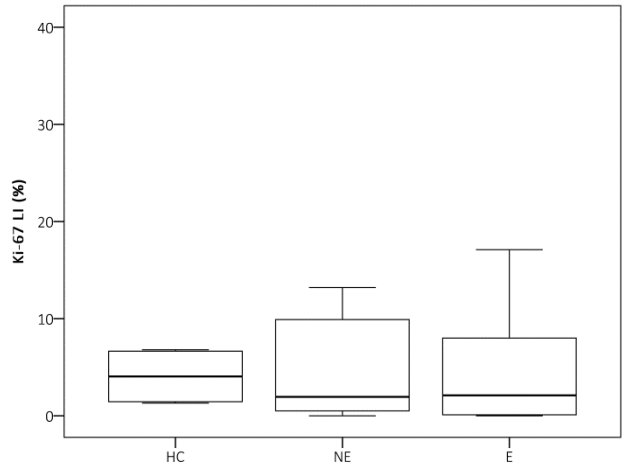

Figure 3. Boxplot of Ki-67 labeling indices. Comparison of the healthy control (HC), the nonenlarged (NE), and the enlarged (E) pituitary groups. No significant differences were observed.

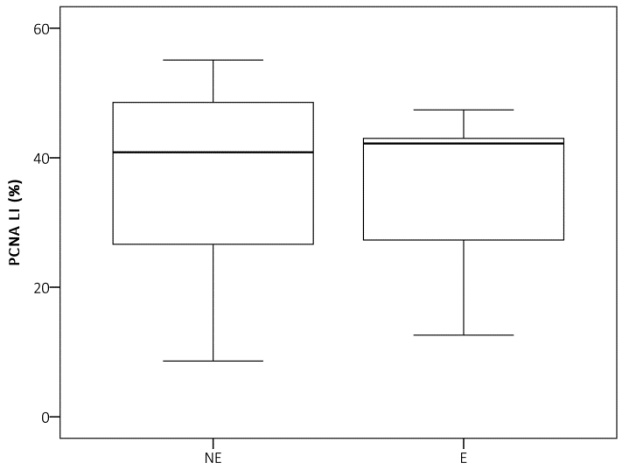

Figure 4. Boxplot of PCNA labeling indices. Comparison of the nonenlarged (NE) and the enlarged (E) pituitary groups. No significant differences were observed.

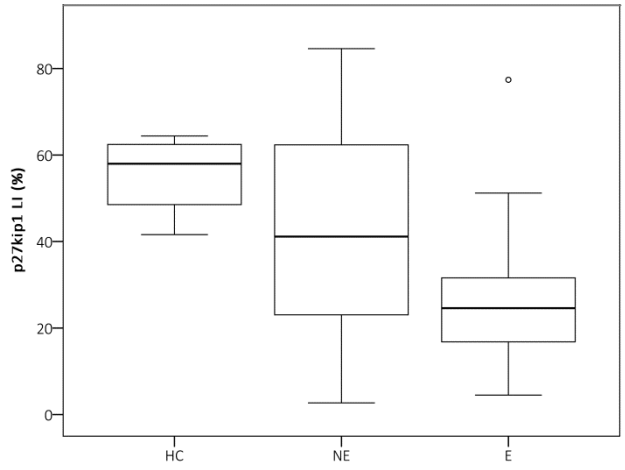

Figure 5. Boxplot of p27kip1 labeling indices. Comparison of the healthy control (HC), the non-enlarged (NE), and the enlarged (E) pituitary groups. A trend towards significance was observed between the enlarged group and the healthy control group (P = 0.05).

Discussion

Pituitary-dependent hypercortisolism is a common endocrinopathy in dogs. Although the pathogenesis of pituitary adenomas has been extensively studied in humans, little is known about the pathogenesis of these tumors in dogs. In this study, we analyzed the expression of the proliferation markers Ki-67 and PCNA and the cell-cycle inhibitor p27kip1 in enlarged and nonenlarged pituitary glands of dogs with PDH. The results indicated that the expression of Ki-67 and PCNA were similar in enlarged and non-enlarged pituitary glands and, for Ki-67, not different from that in control pituitaries, whereas the expression of p27kip1 tended to be lower in enlarged pituitaries than in control pituitaries ($P = 0.05$).

The significance of Ki-67 and PCNA in pituitary adenomas has been strongly debated [10]. PCNA has not been previously used to compare macroadenomas and microadenomas. However, a higher LI was found in recurrent adenomas compared to primary adenomas [16,17]. The usefulness of PCNA as a proliferation marker is questionable, since it seems that it is expressed not only in the cell cycle, but also during other events, for example, DNA repair [27]. Therefore, Ki-67 is used more frequently. Some investigators found significant differences in Ki-67 expression between human corticotroph macroadenomas and microadenomas [7], whereas others did not [19,28,29]. Honneger et al. found Ki-67 expression to be positively correlated with the growth rate of nonfunctional pituitary tumors [10]. In the present study, we did not find Ki-67 expression to be significantly correlated with the P/B ratio. In the recent World Health Organization classification of endocrine neoplasms, the Ki-67 LI is mentioned as a major predictive indicator in pituitary adenomas [30]. However, Losa et al. [31] concluded that in clinically nonfunctioning adenomas, the Ki-67 LI did not contribute to a better regression model for the prognosis of recurrence. Some investigators have found that a high Ki-67 labeling index is associated with tumor invasiveness [11-13], whereas others have not [8,31-33].

p27kip1 knockout mice develop pituitary tumors that originate from corticotroph cells in the intermediate lobe [21-23]. Moreover, a decrease in p27kip1 expression is used as a negative prognostic factor in breast and colon cancer [22,23]. The LI of p27kip1 was found to be lower in adenomatous pituitary tissue than in normal pituitary tissue and is correlated with tumor recurrence [18,23,24]. However, Dahia et al. [21] did not show a correlation between p27kip1 expression and the development of pituitary adenomas in humans. The function of p27kip1 might be regulated at a protein level by differences in phosphorylation [22]. In the present study, this difference was not taken into account because immunohistochemistry does not distinguish between phosphorylated and non-phosphorylated proteins.

Most pituitary adenomas are slow growing and benign. In this study, enlarged pituitaries were included that showed histological signs of malignancy, for example, invasive growth or dedifferentiated, proliferating cells. Furthermore, non-enlarged pituitaries were included harboring corticotroph adenomas, which were identified by histological evaluation. The diagnosis of these so-called microadenomas is not always confirmed with histology, since these small tumors may be lost during surgery or may be absent in the surgical specimen submitted for histopathological examination. The fact that the corticotroph adenomas used in this study were identified at histological examination suggests that they were of sufficient size to be noted, which could have led to a more homogeneous group of patients. This finding may explain why we did not find differences in the LIs between enlarged and non-enlarged pituitaries. We used the P/B ratio to differentiate between enlarged and non-enlarged pituitaries, based on the supposition that the adenoma would cause pituitary enlargement. However, it is possible that pituitary size does not reflect tumor size, and that for this reason, we did not find significant differences in LIs between enlarged and non-enlarged pituitaries. Moreover, in dogs it is often difficult to distinguish adenoma from normal pituitary tissue on imaging (CT or MRI). In enlarged pituitaries, the whole gland is usually homogeneously enhanced, whereas in non-enlarged pituitaries a separate, well defined microadenoma is rarely visible [4,34].

In conclusion, we did not find significant differences between non-enlarged and enlarged pituitaries in their expression of Ki-67 and PCNA. This is further evidence that pituitary adenomas in dogs may behave differently from those in humans. Although canine corticotroph adenomas provide an interesting model to study Cushing's disease, differences between humans and dogs, as recently shown for somatostatin and dopamine receptor expression [35], should be taken into account when using dogs as models of human disease. Apparently, canine cortricotroph adenomas differ from corticotroph adenomas in humans in some of their pathobiological characteristics. In contrast with humans, in dogs Ki-67 and PCNA are not useful as proliferative markers for studying the pathobiology of pituitary corticotroph adenomas. However, a trend toward a lower expression of p27kip1 in enlarged pituitaries compared with control pituitaries was observed, which makes p27kip1 an interesting target for further research.

Acknowledgments

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6 / Expression and clinical relevance of Pax7 and Sox2 in canine corticotroph pituitary adenomas

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Abstract

Pituitary-dependent hypercortisolism (PDH) is a common endocrinopathy in dogs, caused by an adrenocorticotrophic hormone (ACTH) secreting pituitary tumour of the anterior or intermediate lobe. The prognosis of intermediate lobe adenomas is worse than that of anterior lobe adenomas, indicating the possible usefulness of melanotropic markers as prognosticators. Another possible origin of pituitary adenomas is found in cancer stem cells. The aim of the present study was to investigate the expression of melanotroph specific transcription factor paired box protein 7 (Pax7) and stem cell marker and reprogramming factor sex determining region Y-box 2 (Sox2) and relate their expression to clinical parameters.

The mean (± SD) labelling index (LI) for Pax7 was 8.6% ± 21.7% in the adenomas; 1/6 controls had positive staining (LI 15.2%). For Sox2, the LI in the adenomas was 16.9% ± 15.2% and 19.5% ± 11.6% in the controls. Pax7 expression was significantly higher in enlarged pituitaries, compared to non-enlarged pituitaries (P = 0.05), but Pax7 or Sox2 immunopositivity did not correlate to other clinical parameters such as histological diagnosis, survival time or disease-free interval. Gene expression of *PAX7* target genes, such as *proconvertase 2* (*PC2*), *pro-opiomelanocortin* (*POMC*), and *dopamine D2 receptor* (*DRD2*) was significantly lower in the adenoma samples compared to normal tissue, indicating that *PAX7* signalling was not activated in adenomas. It is concluded that Pax7 and Sox2 remain interesting targets for molecular investigations into their role in pituitary tumourigenesis, but are unsuitable as clinical prognosticators in dogs.

Keywords: Canine; Hypercortisolism; Paired box protein 7; Pituitary gland; Sex determining region Y-box 2

Introduction

Pituitary-dependent hypercortisolism (PDH) is a common endocrinopathy in dogs caused by an adrenocorticotrophic hormone (ACTH) secreting tumour in the anterior or intermediate lobe of the pituitary gland [1]. The estimated incidence is 1-2 in 1,000 dogs/year [2]. Research into reliable prognostic factors focuses on pituitary tumourigenesis. Tumours in the intermediate lobe tend to be larger, which is related to a worse prognosis than anterior lobe adenomas [3-5]. This indicates the possible usefulness of melanotropic markers as prognosticators. Given that stem cells can also give rise to pituitary adenomas, stem cell markers may be interesting research targets as prognosticators [6-8].

Previous studies showed that a subset of canine and human corticotroph pituitary adenomas express paired box protein 7 (Pax7), an essential regulator for the melanotroph fate, the predominant cell type of the intermediate lobe [9,10]. Pax7 is a member of the Pax transcription factor family, that is essential in embryonic patterning and postnatal stem cell renewal in many organs [11,12]. It was hypothesised that expression of Pax7 could be related to clinical parameters and as such can be used as prognosticator in dogs with PDH. Pax7 has several melanotroph specific target genes. In mice, *PAX7* inactivation results in a decreased expression of the genes encoding for *pro-opiomelanocortin* (*POMC*), *proconvertase 2* (*PC2*) and *dopamine D2 receptor* (*DRD2)* [9]. The corticotroph and melanotroph cells in the anterior and intermediate lobe of the pituitary gland are derived from the POMC cell lineage, that develops under influence of the pituitary T-box transcription factor Tpit [13]. The prohormone POMC is processed into ACTH in both melanotroph cells and corticotroph cells, followed by further cleavage into α-melanocyte stimulating hormone (α-MSH) by PC2 in melanotroph cells [14]. In human corticotroph adenomas, 47% stained positive for PC2, whereas healthy pituitaries did not [15]. Drd2 is expressed in melanotroph cells and inhibits α-MSH secretion [16]. Approximately 75% of the human corticotroph adenomas express Drd2 [17]. Up regulation of *DRD2* in a pituitary cell line led inhibited cell proliferation and ACTH secretion without a switch to melanotrophic cell type [18].

It is also thought that undifferentiated cells, i.e. stem cells play a role in tumourigenesis in the pituitary gland [6-8]. A possible pituitary stem cell marker is sex determining region Y-box 2 (Sox2), a transcription factor that plays an important role in pituitary development [19]. Mutations in *SOX2* led to developmental abnormalities of the pituitary gland [20]. During organogenesis, Sox2 positive ($Sox2^{+}$) cells are found in Rathke's pouch. Sox2 expression is down regulated in the postnatal pituitary, but a residual level of Sox2 positivity persists and these $Sox2^{+}$ cells show several stem cell characteristics [21-23].

$Sox2^+$ cells do indeed give rise to pituitary adenomas in transgenic mice [23], suggesting Sox2 as a prognostic marker in patients with pituitary adenomas.

The aims of the present study are to analyse the protein and mRNA expression of Pax7 and Sox2 in pituitary tissue removed during hypophysectomy of patients treated for PDH and relate these to clinical parameters, in order to determine whether their expression could serve as a prognostic factor.

Materials and methods

Animals

Pituitary adenoma tissue of client-owned dogs that underwent transsphenoidal hypophysectomy as treatment for PDH [24] was included in the study (n = 58). Clinical characteristics of the dogs are depicted in the appendix (Supplementary Tables S1 and S2). The diagnosis of hypercortisolism was based on an elevated ($\geq 10 \times 10^{-6}$) urinary corticoid-to-creatinine ratio (UCCR), combined with a high dose dexamethasone suppression test and measurement of plasma ACTH concentrations. And further supported by visualisation of the adrenals by ultrasonography and pituitary imaging as previously described [3,25,26]. In dogs with PDH, enlarged pituitaries were distinguished from non-enlarged pituitaries by their pituitary height/brain area (P/B) value [27]. Remission was defined as UCCR $<10 \times 10^{-6}$ and resolution of clinical signs of hypercortisolism. Recurrence was defined as UCCR $\geq 10 \times 10^{-6}$ and reappearance of clinical signs of hypercortisolism after initial remission.

As control tissue, anterior lobes of normal pituitary glands were obtained from five healthy Labrador Retrievers, 11 Beagles and nine Crossbred dogs euthanased in other, unrelated experiments, approved by the Ethics Committee on Animal Experimentation, Utrecht University, the Netherlands, in accordance to the 3R-policy (DEC-number 2007.III.06.080). One normal pituitary gland was obtained from a healthy 8 month old male dog. This dog was a client-owned Bouvier des Flandres euthanased because of spinal trauma in which autopsy was performed after owner's consent. of spinal trauma in which autopsy was performed after owner's consent.

Immunohistochemistry

Specimens of pituitary tissue removed during surgery (n = 44) or autopsy (n = 6) were fixed in 4% neutral buffered formaldehyde, embedded in paraffin, and consecutive sections were used for histology and immunohistochemistry. The diagnosis of pituitary adenoma was made on haematoxylin and eosin (HE) stained tissue sections by a board

certified veterinary pathologist. The diagnosis of corticotroph adenoma was confirmed by immunostaining for ACTH, α-MSH, and growth hormone (GH) as previously described [28].

For the detection of Pax7 and Sox2, 4 µm thick paraffin embedded tissue sections were mounted on poly-L-lysine-coated slides. After deparaffinisation, antigen retrieval was performed by incubation in a citrate bath during 60 min for Pax7 immunostaining and 30 min for Sox2 immunostaining. Endogenous peroxidase activity was blocked by 30 min incubation in a solution of 0.3% H_2O_2 in methanol followed by 30 min treatment with 10% normal goat serum. Incubation with the primary antibodies took place overnight at 4 °C (anti-Pax7 monoclonal mouse antibody, Developmental Studies Hybridoma Bank, isotype IgG1, 1:20, anti-Sox2 monoclonal rabbit antibody, Cell Signaling Technology, isotype IgG, 1:800), followed by a 30 min incubation with secondary antibodies (labelled polymer-HRP anti-mouse for Pax7 and anti-rabbit for Sox2 immunostaining, Dako Envision Systems). Staining was detected with DAB-substrate (Vector laboratories) and slides were counterstained for 10 s with haematoxylin staining.

Antibodies were tested for specificity against canine tissue according to the manufacturer's product information. As a positive control, normal canine pituitary tissue was used. Negative controls were obtained by replacing the primary antibody with aspecific isotype controls (mouse IgG1 isotype control, PE labelled, Biolegend, for Pax7 immunostaining and rabbit IgG isotype control, PE labelled, Antibodies online, for Sox2 immunostaining, see Appendix, Supplementary Figs S1 and S2).

For the detection of PC2, an immunohistochemistry protocol was optimised on canine testis tissue (anti-PC2 polyclonal rabbit antibody, Bioss, Isotype IgG, 1:400). However, even with further optimisation trials of the staining by using different the antigen retrieval techniques, no positive staining could be identified on pituitary tissue, both healthy and adenomas.

Scoring of Pax7 and Sox2 immunopositivity

The location of the pituitary corticotroph adenoma in the tissue sections was determined using the HE slides and slides from consecutive sections stained for ACTH, α-MSH, and GH. The adenomatous tissue usually immunostained positive for ACTH and/or α-MSH but negative for GH. In similar tissue sections, the Pax7 and Sox2 positive cells were identified and quantified in digital images of the immunostained adenomatous areas made by a Color View III camera connected to an Olympus BX41 microscope and Cell^B 3.0 software (Olympus Soft Imaging Systems). In the control samples, a representative section of the anterior lobe was photographed and analysed. On average, 1000 cells/sample were counted at 200× magnification, using the cell counter plug-in of the ImageJ Software

package (National Institute of Mental Health). The labelling index (LI) of Pax7 and Sox2 was obtained by dividing the number of cells staining positive for, respectively, Pax7 and Sox2, by the total number of cells counted.

Gene expression analysis

Gene expression analysis was performed in order to determine whether Pax7 signalling was activated. Total RNA was isolated from snap frozen samples (pituitary adenoma tissue, n = 20, or anterior lobe tissue of healthy controls, n = 20), using Qiagen RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA quantity and quality was measured with the Agilent BioAnalyzer 2100 (Agilent). The RNA integrity number (RIN) values were above 7.0, indicating for sufficient RNA quality to perform quantitative polymerase chain reaction (qPCR). cDNA was synthesised using the iScript cDNA Synthesis Kit (Bio-Rad) according to manufacturer's instructions. The qPCR reaction was performed in duplicate on a Bio-Rad I-Cycler (Bio-Rad). For each PCR sample, a total volume of 10 µL was used, containing 5.0 µL IQ SYBR green SuperMix (Bio-Rad), 4.0 µL of 50-fold diluted cDNA, and 100 µM of both forward and reverse primers. For primers that were not previously described, primer sets were developed and tested with a temperature gradient to determine the optimal annealing temperature (Ta). Details of the primers used are depicted in Table 1. To normalise gene expression the reference genes *TATA box binding protein* (*TBP*), *tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta poly-peptide* (*YWHAZ*) and *hydroxymethylbilane synthase* (*HMBS*) were used, previously identified as the most stable reference genes for pituitary and adenoma samples [29] .

Statistical analysis

Statistical analysis was performed with IBM SPSS Statistics for Windows (Version 20.0, IBM). Groups were created based on pituitary size (normal pituitary tissue, non-enlarged pituitary, enlarged pituitary), histological diagnosis (normal pituitary tissue, anterior lobe adenoma, intermediate lobe adenoma) and recurrence status (recurrence or no recurrence). Normality of data was assessed using a Shapiro-Wilk test and parametric or non-parametric tests were used accordingly. To compare LI's and gene expression levels between different groups of adenomas the Student's t test or Mann Whitney U test were used. Correlation coefficients were calculated using a Spearman rho test. Furthermore, in order to determine the prognostic value of the selected markers, survival analysis was performed using Kaplan Meier curves and the Log Rank test was used to assess significance. Bonferroni corrections for multiple comparisons were applied when appropriate. Significance was set at $P < 0.05$.

Table 1. Nucleotide sequences and annealing temperatures (Ta) of primers used in the quantitative PCR analysis.

Gene	Accession number	F/R	Sequence	Exon	Amplicon size	Ta °C
PAX7	XM_005617934	F	5'-AAGGACGGACACTGTGAC- 3'	3	82	58
		R	5'-CTTTCTTCCCGAACTTGATTCTG-3'	4		
SOX2	XM_005639752	F	5'-AACCCCAAGATGCACAACTC-3'	1	152	61
		R	5'-CGGGGCCGGTATTTATAATC-3'	1		
PC2	XM_542880	F	5'-ATTCAACAGAAAGAAGCGGG-3'	3	141	62
		R	5'-TCTGCCACATTCAAATCCAG-3'	4		
DRD2	XM_005619479	F	5'-CCACTCAAGGGCAACTG-3'	5/6	87	61
		R	5'-CCTGTTCACTGGGAAACTC-3'	6		
POMC	XM_844370	F	5'-GCCTGCAAGCCCGACCTCTC-3'	3	178	62
		R	5'-CTCCGCCCGCCGCCACCTCTTCTT-3'	3		
TBP	XM_849432	F	5'-CTATTTCTTGGTGTGCATGAGG-3'	5	96	58
		R	5'-CCTCGGCATTCAGTCTTTTC-3'	5		
YWHAZ	XM_533072	F	5'-CGAAGTTGCTGCTGGTGA-3'	2	94	58
		R	5'-TTGCATTTCCTTTTTGCTGA-3'	3		
HMBS	XM_546491	F	5'-TCACCATCGGAGCATCT-3'	6	112	61
		R	5'-GTTCCCACCACGCTCTTCT-3'	7		

F, forward primer; R, reverse primer; PAX7, paired box protein 7; SOX2, sex determining region Y-box 2; PC2, proconvertase 2; DRD2, Dopamine D2 receptor; POMC, pro-opiomelamocortin; TPB, TATA box binding protein; YWHAZ, tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein, zeta poly-peptide; HMBS, hydroxymethylbilane synthase.

Results

Histology and immunohistochemistry for ACTH, α-MSH, and GH.

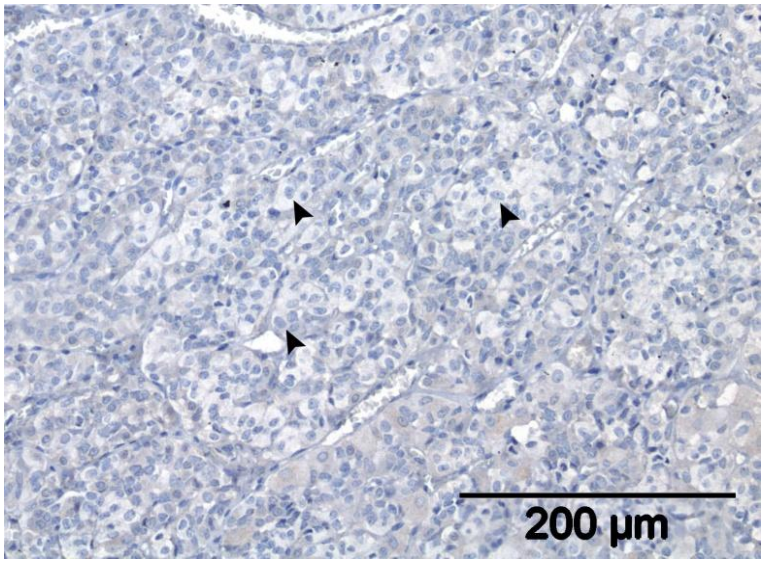
In all dogs with PDH, histological examination of the surgical specimen revealed a corticotroph adenoma staining positive for ACTH and/or α-MSH and mostly negative for GH (See Appendix: Supplementary Table S3). Of the 44 pituitary adenomas used for immunohistochemistry, seven were diagnosed to be intermediate lobe adenomas and 37 were diagnosed as anterior lobe adenomas. In the anterior lobe adenoma group, nine adenomas showed invasive growth or dedifferentiated, proliferating cells on histology. Of the 20 pituitary adenomas used for gene expression analysis, 19 were diagnosed as anterior lobe adenomas and one originated from the intermediate lobe.

Scoring for Pax7 and Sox2

The mean (± standard deviation, SD) LI for Pax7 was 8.6% ± 21.7% in the adenoma samples and positive staining was found in 1/6 control samples (LI 15.2%). For Sox2, the LI

was 16.9% ± 15.2% in adenomas and 19.5% ± 11.6% in controls (Fig. 1, 2, Table S3).

A.



B.

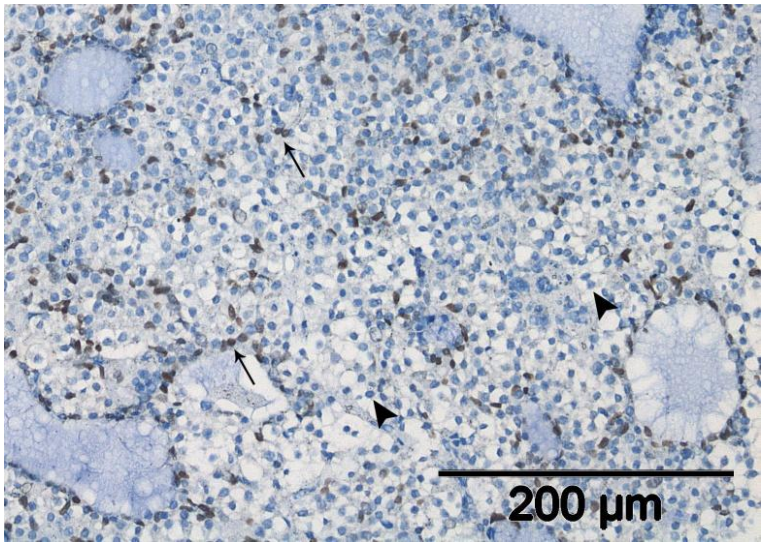
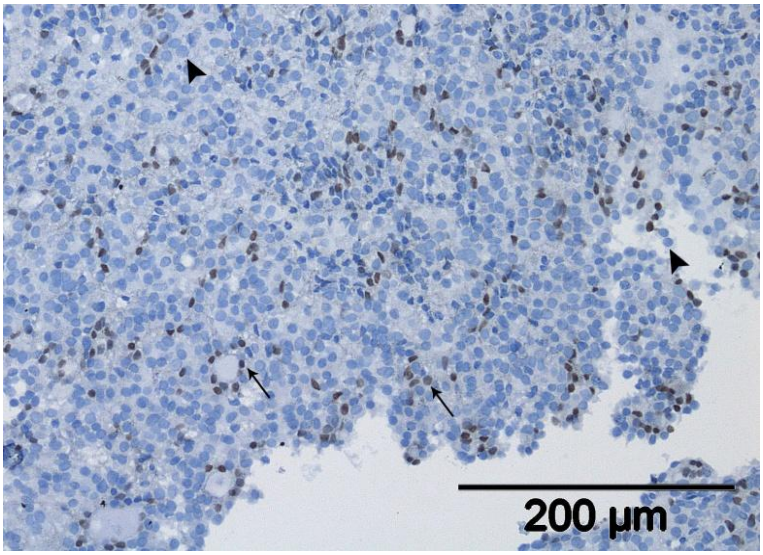


Figure 1. Pax7 immunohistochemistry in (A) pituitary adenoma and (B) the anterior lobe of a normal pituitary gland. Positive nuclei stain brown (arrows), whereas negative nuclei remain blue (arrowheads).

A.



B.

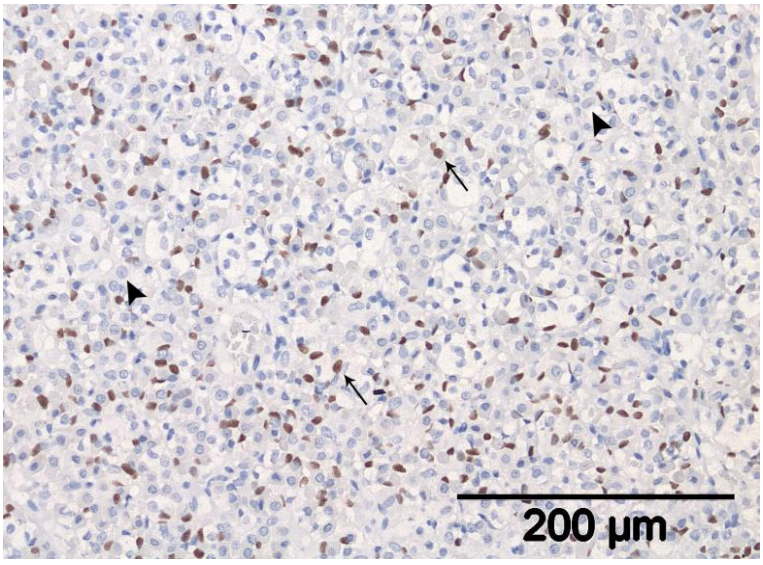


Figure 2. Sox2 immunohistochemistry in (A) a pituitary adenoma and (B) the anterior lobe of a normal pituitary gland. Positive nuclei stain brown (arrows), whereas negative nuclei remain blue (arrowheads).

A significantly higher expression of Pax7 was found in enlarged pituitaries, compared to non-enlarged pituitaries (P=0.05). No other significant differences in Pax7 and Sox2 LI were found taken into consideration either the pituitary size, histopathological diagnosis or recurrence status (Fig. 3).

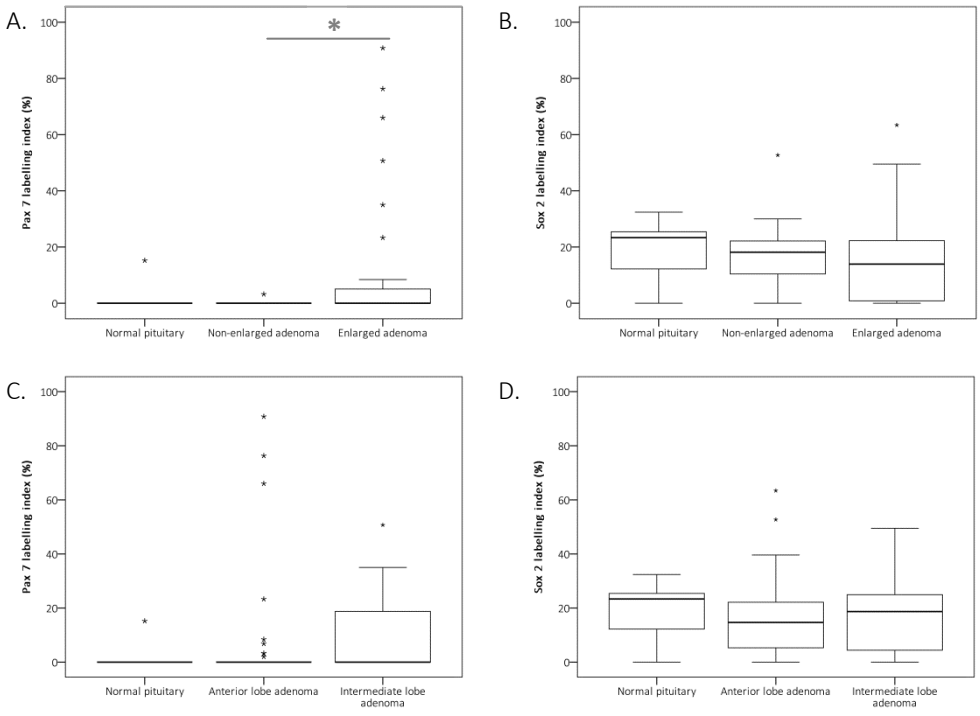


*Figure 3. Boxplots of Pax7 and Sox2 labelling indices. A,B. Comparison of the normal, the non-enlarged, and the enlarged pituitary groups. * P = 0.05. C,D. Comparison of the normal pituitary, the anterior lobe adenoma and the intermediate lobe adenoma groups. No significant differences were observed. Small stars indicate outliers.*

Correlation with clinical signs

Kaplan-Meier curves were plotted for patients with a positive Pax7 LI and with a Pax7 LI of zero (Fig. 4). No significant differences in survival (P = 0.12) or disease free interval (P = 0.95) were found. Also, survival time and disease-free interval were compared between patients with a Sox2 LI higher than the median of 17%, and patients with a Sox2 LI lower than the median of 17% (Fig. 4). The disease free interval of patients with a high Sox2 LI

had a trend towards being longer (P = 0.095), no significant difference in survival was found (P = 0.44).

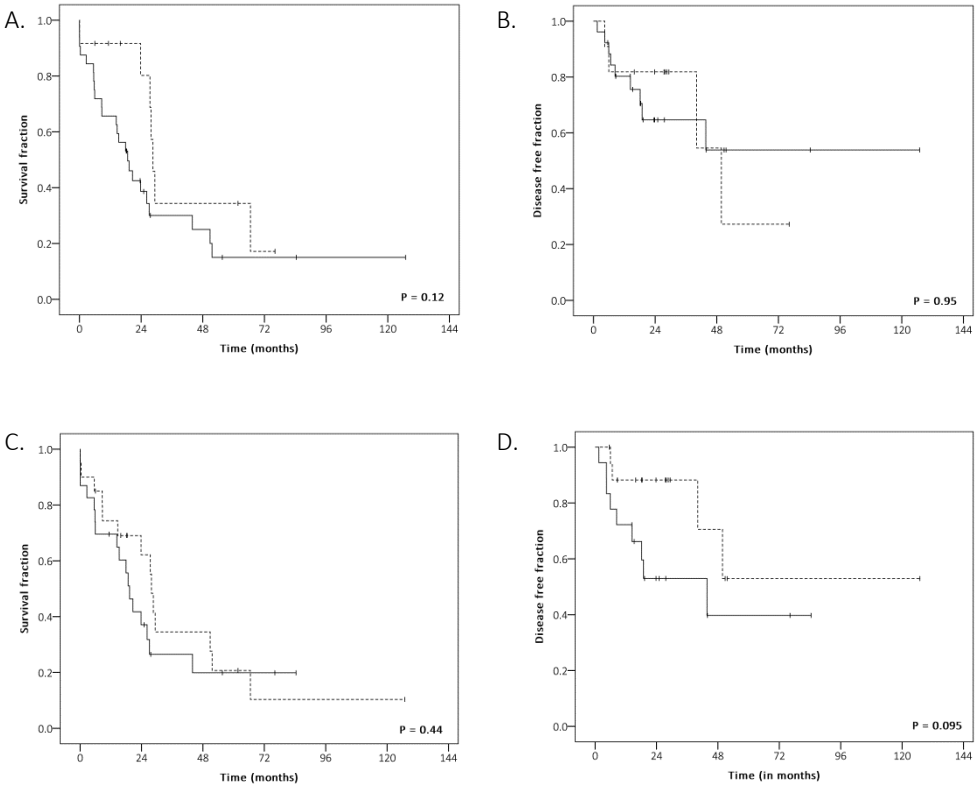


Figure 4. Kaplan Meier survival curves. Survival time (A) and disease free interval (B) in months, comparing Pax7 positive samples (dotted line) with samples with Pax7 Labelling Index (LI) = 0 (continuous line). Survival time (C) and disease free interval (D) in months, comparing Sox2 Labelling Index (LI) <17% (continuous line) with Sox2 LI > 17% (dotted line). Vertical bars indicate censored cases.

Gene expression analysis

Relative mRNA expression was significantly lower in adenoma samples compared to normal pituitary tissue for *PAX7*, *SOX2*, *PC2*, and *DRD2*. No significant difference was found for *POMC* (Fig. 5). There were no significant differences when comparing expression in the groups with and without recurrence. In the controls, a significant

correlation was found between *PAX7* and *PC2* expression (ρ = 0.74, P < 0.001) and between *PAX7* and *POMC* (ρ = 0.51, P = 0.03), no significant correlation was found between *PAX7* and *SOX2*, *PAX7* and *DRD2*, *POMC* and *DRD2*, *POMC* and *PC2* and between *DRD2* and *PC2*. In the adenomas, a significant correlation was found between *PAX7* and *SOX2* (ρ = 0.57, P = 0.01), *PAX7* and *DRD2* (ρ = 0.61, P = 0.01), and between *DRD2* and *PC2* (ρ = 0.74, P < 0.001), no significant correlation was found between *PAX7* and *POMC*, *PAX7* and *PC2*, *POMC* and *DRD2*, and between *POMC* and *PC2*.

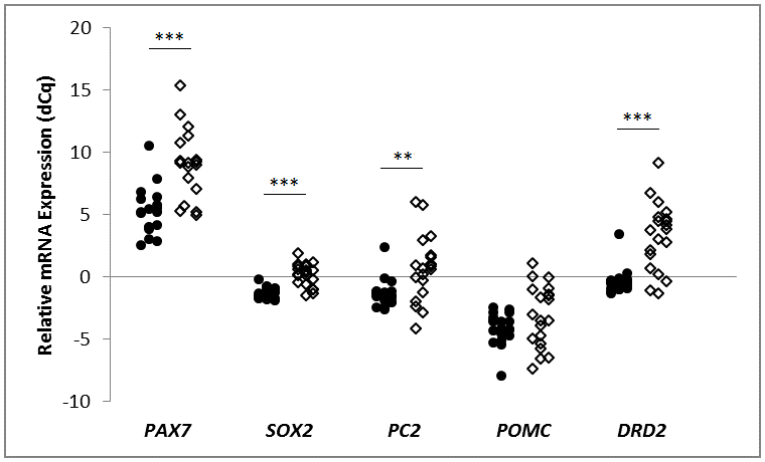


*Figure 5. Dotplot displaying relative mRNA expression of PAX7, SOX2, PC2, POMC and DRD2, normalised by expression of reference genes in adenomas (open diamonds) and controls (solid dots). A higher dCq indicates a lower expression level. *** P<0.001 and ** P<0.01.*

Discussion

In this study, we investigated the expression of transcription factors Pax7 and Sox2, both thought to play a role in the development of the pituitary gland and pituitary tumourigenesis, in canine corticotroph pituitary adenomas. Also, the mRNA expression of several downstream targets of Pax7 was studied to specify the activation status of Pax7 in pituitary adenomas. The results show that there is no significant relation between the expression of these markers and clinical parameters, such as size of the pituitary, histological diagnosis, survival time and disease-free interval.

Pax7 is an essential regulator for the melanotroph fate, limiting Pax7 immunopositivity to the intermediate lobe [9,10]. In the present study, six normal pituitary samples were included. In 5/6 samples, Pax7 expression was limited to the intermediate lobe, however in 1/6 samples, there were also Pax7 positive cells in the anterior lobe (LI, 15.2%). We cannot exclude that this dog had an unrecognised subclinical pituitary pathology. In previous studies, Pax7 expression was found in human and canine corticotroph pituitary adenomas [9,10], suggesting a possible role for Pax7 in the development of these adenomas. It was hypothesised that Pax7 immunopositivity was linked to an intermediate lobe origin of the pituitary adenoma. Since intermediate lobe adenomas tend to be larger, they are thought to have a worse prognosis than anterior lobe adenomas [3-5]. In the present study, the range in Pax7 LI in the pituitary adenomas was very wide, with 69% of samples being negative for Pax7 and others with high LI's. A heterozygous character of canine pituitary adenomas or the possible presence of normal pituitary cells within the adenoma sample might be an explanation of this finding. Previous studies showed that 30% of canine pituitary adenomas expressed Pax7, which was similar to the results of the present study, where 14/44 (31%) of adenoma samples showed Pax7 immunopositivity. We showed a significant higher Pax7 expression in enlarged pituitaries, compared to non-enlarged pituitaries. However, it was not possible to correlate Pax7 immunopositivity to other clinical parameters. This suggests that Pax7 protein expression in pituitary adenoma samples extracted during surgery, is not a useful prognosticator.

Given that immunohistochemistry studies the protein expression of Pax7, immunoreactivity against Pax7 in tissue sections does not necessarily indicate Pax7 activity. The activity of transcription factors is further affected by interaction with other proteins, intracellular localisation, and phosphorylation. Therefore, we studied several target genes of Pax7 with qPCR analysis. Previously, it was shown that Pax7 inactivation resulted in a decreased expression of the genes encoding for *POMC*, *PC2*, and *DRD2* [9]. In the present study, we found that *PAX7*, *PC2* and *DRD2* had a significant lower expression in adenoma tissue, compared to normal pituitary tissue, showing that Pax7 signalling is not activated more in adenomas than in normal pituitary tissue. Although almost half the human corticotroph adenomas in another study were positive for PC2 with immunohistochemistry [15], we were not able to identify PC2 positive cells in the canine pituitary samples, whereas the control tissue (canine testis) stained positive, suggesting a different expression pattern in canine pituitary tissue compared to human corticotroph adenomas. This altogether indicates that Pax7 and its downstream targets may play a species-dependent differential role in pituitary tumourigenesis.

$Sox2^+$ cells show several stem cell characteristics [21-23], which makes it an interesting target in pituitary adenoma research. In the present study, no differential expression of Sox2 could be identified between the studied subgroups, and Sox2 immunopositivity did

not correlate to clinical parameters. Even more so, *SOX2* mRNA expression was significantly lower in adenoma samples compared to normal pituitary tissue. A possible limitation here is the described specific localisation of $Sox2^+$ cells around the pituitary cleft [21,30]. It would be interesting to study the expression of certain markers more specifically in this niche. However, this is not possible with the current study design where most tissue samples were collected during surgery and extracted in small tissue fragments without further determination of the exact regional anatomic origin.

Conclusions

In conclusion, the expression of transcription factors Pax7 and Sox2 in canine corticotroph pituitary adenomas were not differentially expressed in several subgroups of patients, categorized on pituitary size, histological diagnosis and recurrence of disease. Also, Pax7 and Sox2 protein expression did not affect survival time and disease free interval. The gene expression of *PAX7*, *SOX2*, *PC2* and *DRD2* was significantly lower in adenoma samples compared to healthy tissue indicating that Pax7 activity may have a species-dependent differential effect on tumourigenesis. Although Pax7 and Sox2 remain interesting targets for further investigation into their molecular mechanisms of pituitary tumourigenesis, they appear not useful as clinical prognosticators in canine patients with PDH.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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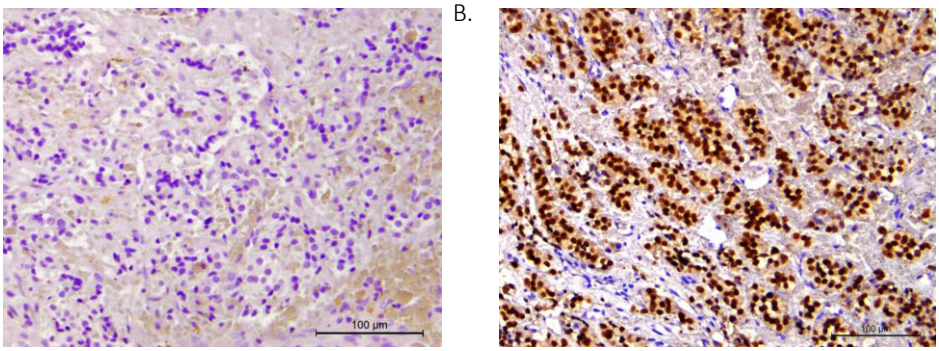
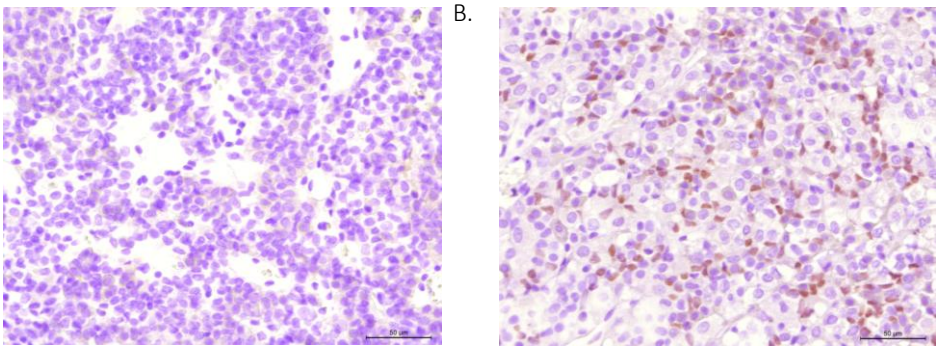
Supplementary data

A. B.



Supplementary Fig 1. Paired homeobox protein 7 (Pax7) immunohistochemistry with (A) a negative isotype control replacing the primary antibody and (B) including the primary antibody directed against Pax7 in a pituitary adenoma. Positive nuclei stain brown, whereas negative nuclei remain blue.

A. B.



Supplementary Fig 2. Sex determining region Y-box 2 (Sox2) immunohistochemistry in (A) a negative isotype control replacing the primary antibody and (B) including the primary antibody directed against Sox2 in the anterior lobe of a normal pituitary gland. Positive nuclei stain brown, whereas negative nuclei remain blue.

Supplementary Table S1. Clinical characteristics of the patients included in the immunohistochemistry study.

No.	Age (y)	Sex	Weight (kg)	Breed	UCCR x 10 $^{-6}$ [a]	Dexamethasone suppression [b]	Pituitary size (H x W x L) [c]	P/B ratio [d]
1	5.2	M	38.0	American Staffordshire Terrier	69.0	93.3	5.6 x 7.2 x 6	0.29
2	9.4	M	8.4	Crossbred	32.0	96.9	3.2 x 4 x 4	0.23
3	7.9	M	8.0	Bolognese	64.5	96.9	3 x 4.2 x 4	0.20
4	6.1	F*	50.0	Bordeaux Dog	36.0	91.4	6 x 9 x 10	0.26
5	6.7	M	8.2	Yorkshire Terrier	42.0	96.7	4 x 4 x 4	0.24
6	3.9	M	22.8	English Cocker Spaniel	64.5	58.1	15 x 17 x 18	0.76
7	10.1	M*	25.2	Crossbred	57.0	94.4	11.5 x 10.6 x 10	0.70
8	8.6	M	33.4	Boxer	44.9	92.4	12.4 x 11.6 x 11	0.70
9	5.8	F	16.7	English Cocker Spaniel	96.1	69.4	14.2 x 17.4 x 17	1.10
10	9.4	F*	19.5	German Shorthaired Pointer	59.0	92.4	7.4 x 8.6 x 8	0.45
11	6.1	M	48.2	Golden Retriever	13.2	87.1	18.3 x 19.6 x 17.2	0.95
12	11.9	F*	23.0	German Wirehaired Pointer	81.5	14.1	7.6 x 8.7 x 11.4	0.47
13	11.7	M*	13.0	Jack Russell Terrier	13.5	74.1	12.6 x 13.4 x 16.2	0.74
14	12.1	M	24.0	Beagle	N/A	N/A	22.5 x 21.3 x 20.7	1.25
15	7.3	M	23.0	Crossbred	0.0	N/A	10.4 x 11 x 11.3	0.65
16	8.3	F*	36.5	Labrador Retriever	30.7	88.3	11.5 x 11.8 x 11.6	0.56
17	8.2	M	23.2	Beagle	74.5	75.8	16.6 x 14.6 x 16	1.00
18	6.5	M	12.8	Crossbred	81.0	60.5	14.9 x 13.8 x 16.1	0.99
19	9.1	F*	18.3	Crossbred	53.5	51.4	6.4 x 6.7 x 7.6	0.41
20	6.9	M	32.9	Crossbred	133.9	89.7	7.5 x 9.1 x 8	0.45
21	9.2	F	36.0	Labrador Retriever	127.7	85.0	4.9 x 5.2 x 6.2	0.29
22	7.4	M*	31.0	Golden Retriever	108.5	84.3	16.5 x 19.7 x 12.4	0.76
23	8.2	F*	31.7	Belgian Shepherd Malinois	32.5	50.8	18.4 x 17.5 x 14.9	1.02
24	10.0	M	21.5	English Staffordshire Terrier	18.1	59.6	11.5 x 12.2 x 10.5	0.64
25	7.0	M	10.4	French Bulldog	417.3	N/A	11.5 x 11.1 x 9.5	0.63
26	2.7	F*	10.7	King Charles Spaniel	100.0	96.0	2 x 6.2 x 4.5	0.29
27	10.7	M*	18.5	Border Collie	55.0	61.8	11.1 x 9.8 x 10.1	0.66
28	8.4	F*	33.2	Boxer	126.6	39.8	10.4 x 12 x 8.1	0.49
29	10.2	M	33.4	Boxer	14.5	84.8	5.8 x 4 x 7	0.27
30	7.1	F*	44.9	American Bulldog	45.5	31.4	13.7 x 15.8 x 14.8	1.38
31	9.2	F*	37.6	Rhodesian Ridgeback	14.8	47.3	13.7 x 13.1 x 13.4	0.78
32	9.7	M	24.8	Crossbred	N/A	N/A	18.9 x 25.6 x 21.6	1.15
33	7.6	F	22.3	Labrador Retriever	N/A	N/A	14.7 x 9.6 x 9.1	0.93
34	9.9	F*	29.1	Labrador Retriever	104.5	4.3	11.4 x 14.5 x 13.2	0.71
35	9.6	M	4.8	Maltese	100.7	97.5	3.4 x 3.8 x 3.2	0.24
36	8.1	F*	12.6	Crossbred	20.5	73.7	19.3 x 15.3 x 15.5	1.40
37	8.9	M	7.4	Australian Terrier	13.9	79.1	4.6 x 5.8 x 5.7	0.30
38	9.3	F*	40.0	Boxer	16.5	52.7	11.7 x 12.4 x 13.9	0.70
39	11.0	M*	10.0	Shih Tzu	N/A	N/A	10.6 x 15 x 12.9	0.67
40	5.6	M	21.0	Beagle	19.6	93.9	4.2 x 5 x 6	0.26
41	5.9	M*	15.8	King Charles Spaniel	383.2	95.1	6.1 x 6.8 x 6.6	0.29
42	10.4	F*	19.7	Beagle	361.5	47.0	7.7 x 7.4 x 9.8	0.48
43	8.4	F*	6.4	Maltese	57.4	95.8	3.6 x 3.8 x 2.2	0.25
44	10.7	M*	25.5	Crossbred	126.0	31.0	17.4 x 17.6 x 21.2	0.74

(Legend to Supplementary Table S1.)

**, castrated; F, female; M, male; N/A, not available; UCCR, urinary corticoid-to-creatinine ratio. [a]Preoperative urinary-to-creatinine ratio (reference < 10×10^{-6}); values are the mean of 2 morning urine samples with a 1-d interval., [b]Preoperative degree of UCCR suppression after high-dose dexamethasone, [c]Pituitary size in mm, as measured on preoperative helical computed tomography, [d]P/B, ratio of the pituitary size and the brain area. P/B ≤ 0.31 indicates a non-enlarged pituitary; P/B > 0.31 indicates an enlarged pituitary.*

Supplementary Table S2. Clinical characteristics and summary of the histopathological diagnosis of the patients included in gene expression analysis.

No.	Age (y)	Sex	BW (kg)	Breed	UCCR $x10^{-6}$ [a]	Dex sup[b]	P/B ratio[c]	Histopathological diagnosis[d]	Immunohistochemistry[e]
1	8.8	M*	24.7	Irish Terrier	29.5	93.6	0.3	Adenoma	N/A
2	8.6	M	33.4	Boxer	44.9	92.4	0.7	Adenoma	N/A
3	8.1	F*	23.0	Crossbred	20.0	80.0	0.4	Adenoma	N/A
4	9.8	M*	8.1	Dachshund	19.8	92.9	0.5	Adenoma	N/A
5	11.7	M*	13.0	Jack Russell Terrier	13.5	74.1	0.7	Adenoma	ACTH +, α-MSH N/A, GH +
6	10.4	F*	3.7	Maltese	213.5	67.2	0.9	Adenoma	ACTH +, α-MSH N/A, GH N/A
7	10.9	F*	8.5	Jack Russell Terrier	26.0	88.1	0.4	Adenoma	ACTH +, αMSH N/A, GH +/-
8	12.1	M	24.0	Beagle	N/A	N/A	1.3	Adenoma	ACTH +, α-MSH +, GH N/A
9	8.7	F*	35.0	Boxer	49.2	98.8	0.5	Adenoma	ACTH +, α-MSH +, GH N/A
10	10.0	F*	28.5	Golden Retriever	31.0	-12.9	0.6	Adenoma	ACTH +, α-MSH +, GH -
11	14.0	M*	21.0	Vizsla	N/A	N/A	1.0	Adenoma	ACTH +, α-MSH +, GH -
12	10.8	F*	31.0	German Pointer	22.1	99.1	0.3	Adenoma	ACTH +, α-MSH +, GH -
13	6.7	F	48.0	Bernese Mountain Dog	100.0	61.0	0.7	Adenoma	ACTH +, α-MSH +/-, GH -
14	9.4	M	34.5	Crossbred	19.7	81.7	0.4	Adenoma	ACTH +, α-MSH +, GH N/A
15	10.4	M*	5.4	Crossbred	62.5	69.6	0.4	Adenoma	ACTH +, α-MSH +, GH N/A
16	8.3	F*	36.5	Labrador Retriever	30.7	88.3	0.6	IL adenoma	N/A
17	5.9	F	24.7	Nova Scotia Duck Toller	82.4	93.7	0.5	Adenoma	ACTH +, α-MSH N/A, GH N/A
18	8.2	M	23.2	Beagle	74.5	75.8	1.0	Adenoma	ACTH -, α-MSH -, GH N/A
19	3.7	M*	6.7	Miniature Pincher	18.6	N/A	0.4	Adenoma	ACTH -, α-MSH -, GH N/A
20	6.5	M	12.8	Crossbred	81.0	60.5	1.0	Adenoma	N/A

**, castrated; BW, Body weight, Dex sup, dexamethasone suppression; F, female; IL, intermediate lobe; M, male; N/A, not available; UCCR, urinary corticoid-to-creatinine ratio, +, marked immunoreactivity, +/-, weak immunoreactivity, -, no immunoreactivity. [a]Preoperative urinary-to-creatinine ratio (reference < 10×10^{-6}); values are the mean of 2 morning urine samples with a 1-d interval., [b]Preoperative degree of UCCR suppression after high-dose dexamethasone, [c]P/B × 10^2 (mm^{-1}), ratio of the pituitary size and the brain area. P/B ≤ 0.31 indicates a non-enlarged pituitary; P/B > 0.31 indicates an enlarged pituitary. [d,e]Diagnosis as stated by a veterinary pathologist based on haematoxylin and eosin staining and immunohistochemistry for ACTH, α-MSH, and GH.*

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Supplementary Table S3. Summary of the histopathological diagnosis, the patients' follow-up, and the labelling indices for Pax7 and Sox2.

Histopathological Diagnosis[a]	Immunohistochemistry[b]	Survival (mo)	DFI (mo)	Rem.[c]	Rec.[d]	LI (%)[e] Pax7	Sox2
Adenoma	N/A	17.9	14.4	yes	yes	0.0	5.9
Adenoma	N/A	23.8	23.8	yes	no	3.2	5.8
Adenoma	N/A	50.8	50.8	yes	no	0.0	23.0
Adenoma	N/A	8.7	8.7	yes	no	0.0	30.0
Adenoma	N/A	126.9*	126.9	yes	no	0.0	18.8
Adenoma	N/A	2.6	1.5	yes	yes	0.0	0.0
Adenoma, mal. char.	N/A	43.9	43.9	yes	no	0.0	0.0
Adenoma	ACTH +, α-MSH +, GH -	23.8	23.8	yes	no	0.0	39.6
Adenoma, mal. char.	N/A	27.1	18.9	yes	yes	0.0	13.7
IL adenoma	ACTH +, α-MSH +, GH -	66.5	49.8	yes	yes	35.0	18.7
Adenoma	ACTH N/A, α-MSH N/A, GH -	15.3	15.3	yes	no	0.0	0.0
Infiltrative adenoma	ACTH +, α-MSH +, GH -	27.4	27.4	yes	no	2.0	30.7
Adenoma	ACTH +, α-MSH N/A, GH +	20.6	8.4	yes	yes	0.0	10.1
Adenoma	ACTH +, α-MSH +, GH N/A	0.0	0.0	no		0.0	0.0
Infiltrative adenoma	ACTH +, α-MSH +, GH N/A	28.6	28.6	yes	no	66.0	63.4
IL adenoma	ACTH +/-, α-MSH +/-, GH -	84.4*	84.4	yes	no	0.0	0.0
Adenoma	ACTH -, α-MSH -, GH N/A	61.7*	40.1	yes	yes	8.4	24.0
Adenoma	ACTH -, α-MSH +, GH -	18.8	4.4	no	yes	0.0	4.8
IL adenoma	ACTH +, α-MSH +, GH -	5.6	5.6	yes	no	0.0	19.2
IL adenoma	ACTH +, α-MSH +, GH -	76.1*	76.2	yes	no	2.5	8.8
Adenoma	ACTH -, α-MSH -, GH +	51.6	51.6	yes	no	0.0	17.5
IL adenoma	ACTH +, α-MSH +, GH -	5.8	0.0	no		0.0	0.0
Adenoma	ACTH +, α-MSH +/- , GH -	11.3*	4.4	yes	yes	6.8	9.7
Adenoma	ACTH +, α-MSH +/- , GH -	19.3	19.3	yes	no	0.0	14.5
Infiltrative adenoma	ACTH +, α-MSH +/-, GH -	6.0*	6.0	yes	yes	3.3	17.4
Infiltrative adenoma	ACTH +, α-MSH +/- , GH -	0.0	0.0	no		0.0	21.3
Adenoma	ACTH +, α-MSH - , GH +	55.6*	43.7	yes	yes	0.0	9.0
Adenoma, mal. char.	ACTH +, en α-MSH + , GH -	14.7	6.7	yes	yes	0.0	28.2
Adenoma	ACTH +, α-MSH - , GH -	14.3	14.3	yes	no	0.0	15.9
Adenoma	N/A	5.5	0.0	no		0.0	1.7
Adenoma	ACTH +, α-MSH + , GH -	27.9	27.9	yes	no	90.8	38.4
Adenoma	ACTH +, α-MSH - , GH -	0.1	0.0	no		76.3	0.0
Infiltrative adenoma	ACTH +, α-MSH +, GH -	29.3	29.3	yes	no	23.3	20.5
Adenoma	ACTH +, α-MSH - , GH -	26.2	18.2	yes	yes	0.0	13.9
Adenoma	ACTH +, α-MSH +, GH -	18.2*	18.2	yes	no	0.0	52.7
Adenoma	ACTH +, α-MSH - , GH -	0.1	0.0	no		0.0	0.0
Adenoma	ACTH +, α-MSH - , GH -	8.7	8.7	yes	no	0.0	19.8
Infiltrative adenoma	ACTH +, α-MSH - , GH -	5.9	5.9	yes	yes	0.0	14.5
Adenoma	ACTH -, α-MSH - , GH -	18.4*	18.4	yes	no	0.0	19.4
Adenoma	ACTH +, α-MSH + , GH -	27.6*	27.6	yes	no	0.0	0.0
Adenoma	ACTH +, α-MSH + , GH -	23.6*	23.6	yes	no	0.0	N/A
IL adenoma	ACTH +, α-MSH +, GH -	15.9*	15.9	yes	no	50.7	49.5
Adenoma	ACTH +, α-MSH + , GH -	25.0*	25.0	yes	no	0.0	14.9
IL adenoma	ACTH +, α-MSH - , GH N/A	0.4	0.0	no		0.0	30.7
Normal pituitary	N/A					0.0	25.4
Normal pituitary	N/A					0.0	12.2
Normal pituitary	N/A					0.0	0.0
Normal pituitary	N/A					0.0	21.5
Normal pituitary	N/A					15.2	32.4
Normal pituitary	N/A					0.0	25.2

(Legend to Supplementary Table S3.)

**, alive; +, marked immunoreactivity, +/-, weak immunoreactivity, -, no immunoreactivity; DFI, disease-free interval, IL, intermediate lobe; LI, labelling index; mal. char., malignant characteristics, N/A, not available; rec., recurrence; rem., remission; [a,b]Diagnosis as stated by a veterinary pathologist based on haematoxylin and eosin staining and immunohistochemistry for ACTH, α-MSH, and GH. [c]Remission was defined as UCCR < 10 x 10^{-6} and resolution of clinical signs of hypercortisolism. [d]Recurrence was defined as UCCR ≥ 10 x 10^{-6} and return of clinical signs and symptoms of hypercortisolism after initial remission. [e]Labelling index: number of positive cells/total number of cells counted.*

7 / Expression stability of reference genes for quantitative-RT-PCR of healthy and diseased pituitary tissue samples varies between humans, mice, and dogs

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Abstract

Pituitary surgery generates pituitary tissue for histology, immunohistochemistry, and molecular biological research. In the last decade, the pathogenesis of pituitary adenomas has been extensively studied in humans, and to a lesser degree in dogs, and tumor oncogenesis has been studied in knock-out mice, often by means of quantitative reversed-transcriptase PCR (RT-qPCR). A precondition of such analyses is that so-called reference genes are stably expressed regardless of changes in disease status or treatment. In this study, the expression of six frequently used reference genes, namely, *tata box binding protein* (*tbp*), *tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide* (*ywhaz*), *hydroxymethylbilane synthase* (*hmbs*), *beta-2-microglobulin* (*b2m*), *succinate dehydrogenase complex subunit A* (*sdha*), and *glyceraldehyde 3 phosphate dehydrogenase 1* (*gapdh*)), was studied in pituitary tissue (normal and adenoma) from three species (humans, mice, and dogs). The stability of expression of these reference genes differed between species and between healthy and diseased tissue within one species. Quantitative analysis based on a single reference gene that is assumed to be stably expressed might lead to wrong conclusions. This cross-species analysis clearly emphasizes the need to evaluate the expression stability of reference genes as a standard and integral aspect of study design and data analysis, in order to improve the validity of the conclusions drawn on the basis of quantitative molecular analyses.

Keywords: Reference genes, pituitary, quantitative-RT-PCR

Introduction

The anterior lobe of the pituitary gland consists of five different hormone-producing cells, i.e. corticotrophs, thyrotrophs, gonadotrophs, somatotrophs, and lactotrophs [1,2]. Pituitary adenomas arising from these cell types may be endocrine active adenomas or clinically nonfunctioning adenomas [3]. Somatotroph adenomas secreting growth hormone (GH) cause acromegaly, and corticotroph adenomas secreting adrenocorticotropic hormone (ACTH) cause Cushing's disease [1,4]. The prevalence of pituitary adenomas in humans was recently found to be much higher than previously reported, with an incidence of 1 in 1,064–1,289, but Cushing's disease remains rare [5,6]. In contrast, the estimated prevalence of pituitary adenomas causing Cushing's disease in dogs is higher, 1 or 2 in 1000 [3,7,8]. Surgery is the treatment of choice for Cushing's disease in humans [22], and hypophysectomy is routinely used in the Netherlands for treatment of Cushing's disease in dogs [7,9]. Pituitary surgery generates pituitary tissue for histology, immunohistochemistry, and molecular biological research. In the last decade, the pathogenesis of pituitary adenomas has been extensively studied in humans, and to a lesser degree in the dogs. Genes or transcription factors identified in this way as being relevant to pituitary oncogenesis can be tested in experimental knock-out or transgenic mouse models [10,11] or cell lines [12]. The Rb knock-out mouse model has been widely used in pituitary adenoma research, since heterozygous mice develop pituitary adenomas [13]. Now that the sequences of the human [14] and canine [15] genome are known, it is possible to compare human and dog pituitary adenomas at the molecular level, to get a better understanding of the mechanisms involved.

Quantitative reversed-transcriptase PCR (RT-qPCR) lends itself to the investigation of tumor pathogenesis and the response to treatment, having a high sensitivity and specificity, although small variations in input quality and quantity will greatly affect the interpretation of data. MIQE-precise guidelines have been established to standardize the experimental design, methodology, and data interpretation of real-time PCR experiments [16]. An important element of data interpretation is the stability of expression of so-called reference genes, the expression of which should be stable regardless of changes in diseases status or treatment. For a number of years, investigators have been aware that the expression stability of several frequently used reference genes, such as beta-actin or gapdh, is far more variable than tacitly assumed [17]. Consequently, the most stable reference genes for a specific organ in a specific situation are not necessarily one-to-one transferable to other species. This prompted us to investigate the expression stability of six frequently used reference genes, namely, *tata box binding protein* (*tbp*), *tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide* (*ywhaz*), *hydroxymethylbilane synthase* (*hmbs*), *beta-2-microglobulin* (*b2m*), *succinate*

dehydrogenase complex subunit A (*sdha*), and *glyceraldehyde 3 phosphate dehydrogenase 1* (*gapdh*)), in three species (humans, mice, and dogs) used in pituitary research [18-22]. We analyzed the expression stability of reference genes in human, canine, and murine samples of normal and diseased (adenoma) pituitaries, to determine which reference genes or combination of references genes should be used for valid data interpretation.

Materials and Methods

Tissue sampling

Humans

Pituitary tumors were diagnosed on the basis of clinical biochemistry of blood, hormonal and urine parameters and magnetic resonance imaging, as previously described [23]. In all patients, the pituitary tumor was removed by a transsphenoidal approach; samples were taken and frozen on dry ice and stored at -80 °C until assayed [24]. Tumor specimens included 5 prolactinomas, 5 non-functioning adenomas, 4 corticotroph adenomas, 5 somatotroph adenomas, and 2 samples of normal pituitary tissue collected at autopsy. Different tumor types were not analyzed separately.

Mice

Five Rb wild-type (WT) mice and 14 Rb +/- mice [25] were gas euthanized in experiments approved by the Ethics Committee on Animal Experimentation of the Faculty of Veterinary Medicine, Utrecht University, the Netherlands. The genotype of mice was determined by PCR as described previously [25]. The mice were euthanized due to the consequences of pituitary tumor expansion with neurological signs such as circling, anorexia and wasting, which was defined as an humane endpoint for the animal experiment. Immediately after death, the mice were decapitated, and the skull was opened under 3.3 x magnification. The brain was lifted from the skull, turned upside down, and the pituitary gland (Rb WT) or macroscopic pituitary tumors (Rb +/-) were removed, snap frozen in liquid nitrogen, and stored at -70°C until assay.

Dogs

Samples of pituitary adenomas were collected from 20 dogs with confirmed Cushing's disease during transsphenoidal hypophysectomy. The diagnosis was established by clinical biochemistry of blood, hormonal and urine parameters, computed tomography of the pituitary gland and ultrasonography of the adrenal glands, as described previously [7,26]. Tissue was snap frozen in liquid nitrogen and stored at -70°C until assay. Normal pituitary tissue was obtained as surplus material from 20 dogs that were euthanized in non-related experiments approved by the Ethics Committee on Animal Experimentation of the Faculty

of Veterinary Medicine, Utrecht University, the Netherlands. Immediately after death, the brain was removed, and the pituitary gland was detached, snap frozen in liquid nitrogen, and stored at -70°C until assay. Before RNA isolation, the anterior lobe of the pituitary gland was separated from the neurointermediate lobe under 3.3 x magnification with a No. 10 scalpel. Experiments were conducted with the anterior lobe tissue.

RNA isolation, reversed transcription, and quantitative-RT-PCR

Total RNA was isolated from snap frozen samples, using Qiagen RNeasy Mini Kit (Qiagen, Leusden, the Netherlands) according to the manufacturer's instructions. A 15-min on-column DNase-I treatment was included to minimize genomic DNA contamination. RNA quantity was evaluated spectrophotometrically using Nanodrop ND-1000 (Isofen Life Sciences, IJsselstein, the Netherlands) and RNA quality was analyzed with the Agilent BioAnalyzer 2100 (Agilent, Palo Alto, CA). Obtained RNA yields were 7-823 ng/µl in the murine samples, 26-680 ng/µl in the human samples and 45-805 ng/µl in the canine samples. All RIN values were above 7.0, indicating high-quality RNA. Reverse transcription was performed with 100 ng of total RNA in a total volume of 20 µL using iScript™ cDNA Synthesis Kit (Bio-rad, Veenendaal, the Netherlands) containing a mix of both random hexamer and oligo-dT primers.

The RT-qPCR reaction was performed in an iCylcer iQ™ (Bio-Rad) fitted with a MyiQ™ Single-Color Real-Time PCR Detection System (Bio-Rad) with 1 µL template. Reactions further contained 1 µL of 5x diluted cDNA, 12.5 µl IQ SYBRGreen SuperMix (Bio-Rad), and 400 nM of each primer (Eurogentec, Maastricht, the Netherlands, Table 1) in a reaction volume of 25 µL. Details of the primers and intron spanning to reduce amplification of genomic DNA are depicted in Table 1. For primers that were not previously described, primer sets were developed using known genome sequences available from Ensembl (www.ensembl.org) or GenBank (www.ncbi.nih.gov/genbank/index.html). Primer design was performed with Oligo Explorer 1.1.0 software (www.genelink.com/tools/gl-downloads.asp). To prevent amplification of genomic DNA, the primers were positioned in different exons. Uniqueness and specificity of each primer were verified using the Basic Local Alignment Search Tool (www.ncbi.nlm.nih.gov/blast) returning GenBank Accession Numbers.

*Table 1. Primers used for RT-qPCR of the target genes tata box binding protein (tbp), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (ywhaz), hydroxymethylbilane synthase (hmbs), beta-2-microglobulin (b2m) , succinate dehydrogenase complex subunit A (sdha), and glyceraldehyde 3 phosphate dehydrogenase 1 (gapdh). * NCBI mRNA reference sequence.*

Species	Gene	Forward primer 5'-3'	Exon	Reverse primer 5'-3'	Exon	Amplicon Size	$Temp_a$ (°C)	Accession # *
Human	*tbp*	TGCACAGGAGCCAAGAGTGAA	5	CACATCACAGCTCCCCACCA	7	132	63.5	NM_003194
	ywhaz	ACTTTTGGTACATTGTGGCTTCAA	10	CCGCCAGGACAAACCAGTAT	10	94	64	NM_003406
	hmbs	GGCAATGCGGCTGCAA	1	GGGTACCCACGCGAATCAC	3	64	56	NM_000190
	b2m	CTTTGTCACAGCCCAAGATAG	4	CAATCCAAATGCGGCATCTTC	4	83	58	NM_004048
	sdha	TGGGAACAAGAGGGCATCTG	3	CCACCACTGCATCAAATTCATG	3	86	58	NM_004168
	gapdh	TGCACCACCAACTGCTTAGC	8	GGCATGGACTGTGGTCATGAG	8/9	87	62	NM_002046
Mouse	*tbp*	GGCCTCTCAGAAGCATCACTA	2/3	GCCAAGCCCTGAGCATAA	3	167	66	NM_013684
	ywhaz	AACAGCTTTCGATGAAGCCAT	3/4	TGGGTATCCGATGTCCACAAT	4/5	120	64	NM_011740
	hmbs	ACTCTGCTTCGCTGCATT	11	AGTTGCCCATCTTTCATCACTG	12/13	101	58	NM_013551
	b2m	TTCTGGTGCTTGTCTCACTGA	1	CAGTATGTTCGGCTTCCCATTC	2	104	64	NM_009735
	sdha	GGAACACTCCAAAAACAGACCT	2	CCACCACTGGGTATTGAGTAGAA	2/3	106	57	NM_023281
	gapdh	GGAGTCCACTGGCGTCTTCAC	5	GAGGCATTGCTGATGATCTTGAGG	6/7	165	58	NM_008084
Dog	*tbp*	CTATTTCTTGGTGTGCATGAGG	5	CCTCGGCATTCAGTCTTTTC	5	96	57	XM_849432
	ywhaz	CGAAGTTGCTGCTGGTGA	2	TTGCATTTCCTTTTTGCTGA	2/3	94	58	XM_533072
	hmbs	TCACCATCGGAGCATCT	6	GTTCCCACCACGCTCTTCT	6/7	112	61	XM_546491
	b2m	TCCTCATCCTCCTCGCT	1	TTCTCTGCTGGGTGTCG	2	85	61.2	XM_535458
	sdha	GCCTTGGATCTCTTGATGGA	6	TTCTTGGCTCTTATGCGATG	6	92	61	XM_535807
	gapdh	TGTCCCCACCCCCAATGTATC	2	CTCCGATGCCTGCTTCACTACCTT	2	100	58	NM_001003142

Mfold (www.bioinfo.rpi.edu/applications/mfold) was used to determine the formation of secondary structures in the formed product. Cycling conditions were 3 min at 95°C, followed by 45 cycles with denaturing template for 20 s at 95°C, followed by 30 s at melting temperature (Tm), and elongation at 72°C for 30 s. When the annealing temperature (Ta) was higher than 57°C, the elongation step at 72°C was omitted and extension took place at Ta. Subsequently, a melt curve, to verify amplification of a single product, was generated starting at 65°C and increasing to 99°C by 1°C for each 30-s cycle. The amplification efficiency was always between 92% and 107%, and sequencing of the amplicon products confirmed product specificity. Contamination of RNA with genomic DNA was verified with minus-RT controls; no-template controls were included to test for other contaminations.

Data analysis

Data were analyzed using GeNorm [27] and NormFinder [28]. GeNorm (part of qBase+ software, Biogazelle, Ghent, Belgium, www.biogazelle.com) calculates the stability of expression (M) of one gene based on the average pair-wise variation between all the studied reference genes. The highest M values are for genes with the least stable expression, suggesting a less than optimal reference gene candidate. Step-by-step elimination of the least stable genes generates a ranking of reference genes according to their M values and ultimately results in the identification of the most stable genes [29]. GeNorm can subsequently be used to determine the optimal number of reference genes. The pair-wise variation between these genes defines V. The lower V, the smaller the variation. For example, V3/4 indicates the variation in normalization factor with 3 vs. 4 reference genes. A cut-off value of 0.15 for the pairwise variation was chosen, indicating that the use of a set of reference genes with a pairwise variation < 0.15 results in valid normalization [16,30]. Statistical analysis was performed with R software (Version 2.12.0, Free Software Foundation).

NormFinder analysis consists of a model-based approach, by which the overall variation in expression within sample groups of interest (intragroup variation) and the variation across the sample population groups (intergroup variation) are calculated for each evaluated reference gene. The combination of the two variation parameters results in a stability value, which represents a practical measure of the systematic error that will be introduced when using a particular reference gene.
NormFinder calculations were done using the NormFinder plug-in for Microsoft Excel (www.mdl.dk/publicationsnormfinder.htm).

Results

The ranking according to both GeNorm and Normfinder analyses is depicted in Tables 2 and 3. In the human samples, GeNorm and Normfinder analyses revealed that *gapdh*, *tbp*, *sdha*, and *ywhaz* were the most stable reference genes overall and *b2m* and *hmbs* the least stable. Despite differences in the number of healthy (n=2) and tumor (n=19) samples, the ranking per healthy or tumor group was similar, although the ranking of gapdh was lower with GeNorm analysis than with Normfinder analysis. The cut-off value of 0.15 for the pairwise variation (V) was not reached in the tumor group and in the combined (healthy plus diseased tissue) group, whereas 2 or 3 reference genes were found sufficient for reliable normalization in the normal pituitary samples (Figure 1).

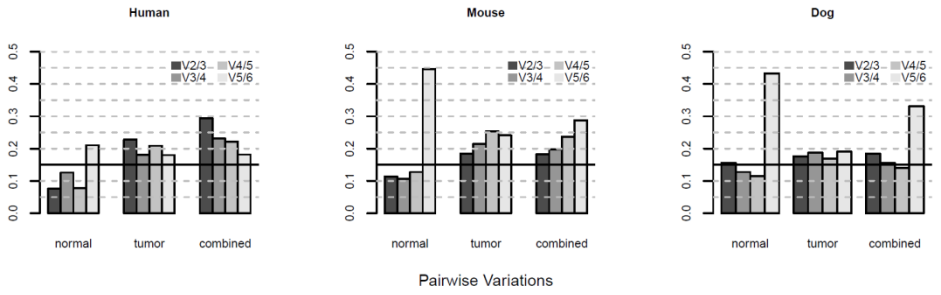

Figure 1. Determination of the optimal number of reference genes for normalization. The GeNorm program calculates the normalization factor by assessing the optimal number of reference genes for generating the M factor by calculating the pair-wise variation V. The pair-wise variation between these genes defines V [29]. The lower V, the less the variation. V3/4 indicates the variation in normalization factor with 3 vs. 4 reference genes.

In the murine samples, *sdha*, *ywhaz*, *hmbs*, and *b2m* were the most stable reference genes in both GeNorm and Normfinder analyses. In the normal murine pituitary samples, *b2m*, *hmbs*, *gapdh*, and *ywhaz* ranked the highest on GeNorm analysis, whereas *ywhaz*, *sdha*, *hmbs* and *tbp* ranked the highest on Normfinder analysis. In the pituitary tumor samples, the most stably expressed genes were *sdha*, *ywhaz*, *b2m*, and *hmbs* with both analytical methods. The cut-off value of 0.15 for the pairwise variation was not reached in the tumor and combined groups, whereas 2 or 3 reference genes were found sufficient for reliable normalization in the normal pituitary samples.

In the canine samples, GeNorm analysis showed *tbp*, *ywhaz*, *hmbs* and *b2m* to be the most stable reference genes in the combined group, whereas Normfinder showed *ywhaz*,

tbp, *hmbs* and *sdha* to be the most stable. For the normal pituitary samples, *tbp*, *ywhaz*, *sdha* and *ywhaz* were the most stable reference genes with both analytical methods, although the ranking order differed slightly. The most stable genes in the pituitary tumor samples were *hmbs*, *ywhaz*, *tbp*, and *b2m* with GeNorm and *tbp*, *ywhaz*, *hmbs*, and *sdha* with NormFinder. Analysis of pairwise variations showed that 3 or 4 reference genes were sufficient for reliable normalization in the normal pituitary samples and overall.

Table 2. Ranking of the stability of expression of reference genes based on GeNorm and NormFinder analysis using human, murine, and canine samples. The highest rank is the most stably expressed gene. G = GeNorm ranking, N = Normfinder ranking.

Human						
	Normal		Tumor		Combined	
Rank	G	N	G	N	G	N
1	*hmbs*	*sdha*	*sdha*	*tbp*	*gapdh*	*gapdh*
2	*tbp*	*ywhaz*	*tbp*	*gapdh*	*tbp*	*tbp*
3	*sdha*	*gapdh*	*hmbs*	*hmbs*	*sdha*	*ywhaz*
4	*ywhaz*	*hmbs*	*gapdh*	*sdha*	*ywhaz*	*sdha*
5	*gapdh*	*tbp*	*ywhaz*	*ywhaz*	*hmbs*	*hmbs*
6	*b2m*	*b2m*	*b2m*	*b2m*	*b2m*	*b2m*

Mouse						
	Normal		Tumor		Combined	
Rank	G	N	G	N	G	N
1	*b2m*	*ywhaz*	*sdha*	*ywhaz*	*sdha*	*sdha*
2	*hmbs*	*sdha*	*ywhaz*	*sdha*	*ywhaz*	*ywhaz*
3	*gapdh*	*hmbs*	*b2m*	*hmbs*	*b2m*	*hmbs*
4	*ywhaz*	*tbp*	*hmbs*	*b2m*	*hmbs*	*b2m*
5	*sdha*	*b2m*	*tbp*	*gapdh*	*gapdh*	*gapdh*
6	*tbp*	*gapdh*	*gapdh*	*tbp*	*tbp*	*tbp*

Dog						
	Normal		Tumor		Combined	
Rank	G	N	G	N	G	N
1	*tbp*	*ywhaz*	*hmbs*	*tbp*	*tbp*	*ywhaz*
2	*ywhaz*	*b2m*	*ywhaz*	*ywhaz*	*ywhaz*	*tbp*
3	*sdha*	*tbp*	*tbp*	*hmbs*	*hmbs*	*hmbs*
4	*b2m*	*sdha*	*b2m*	*sdha*	*b2m*	*sdha*
5	*hmbs*	*hmbs*	*sdha*	*b2m*	*sdha*	*b2m*
6	*gapdh*	*gapdh*	*gapdh*	*gapdh*	*gapdh*	*gapdh*

Table 3. Comparison of ranking of the reference genes in combined healthy and diseased pituitary samples from humans, mice, and dogs. The highest rank is the most stably expressed gene. G = GeNorm ranking, N = Normfinder ranking.

	Human		Mouse		Dog	
Rank	G	N	G	N	G	N
1	*gapdh*	*gapdh*	*sdha*	*sdha*	*tbp*	*ywhaz*
2	*tbp*	*tbp*	*ywhaz*	*ywhaz*	*ywhaz*	*tbp*
3	*sdha*	*ywhaz*	*b2m*	*hmbs*	*hmbs*	*hmbs*
4	*ywhaz*	*sdha*	*hmbs*	*b2m*	*b2m*	*sdha*
5	*hmbs*	*hmbs*	*gapdh*	*gapdh*	*sdha*	*b2m*
6	*b2m*	*b2m*	*tbp*	*tbp*	*gapdh*	*gapdh*

Discussion

RT-qPCR is a widely used to analyze gene expression. The major advantages of the technique are its high sensitivity and specificity, but small variations in input quality and quantity greatly affect data interpretation. According to recently published guidelines [16], data interpretation relies strongly on the stability of expression of reference genes. The most stable reference genes for one organ and species are not necessarily the same in other organs or species, as shown in our previous study [31]. The aim of the present study was to determine which combination of reference genes should be used to investigate pituitary samples from humans, mice, and dogs., in order to be able to interpret findings correctly.

The most stable reference genes in normal and tumor pituitary samples were *gapdh*, *tbp*, *sdha* and *ywhaz* for human tissue; *sdha*, *ywhaz*, *b2m*, and *hmbs* for murine tissue; and *tbp*, *ywhaz*, *hmbs*, and *b2m* for canine tissue. Many published studies used a single reference gene for normalization [18,32,33], but this is not a valid method for accurate normalization, because no single gene has a constant level of expression in all tissue samples [16]. Lisowski et al showed this in bovine samples, where the expression stability of reference genes in pituitary samples differed from that of the same genes in liver, kidney, and thyroid samples. For example, the frequently used reference gene *ACTB* was shown to be unstable in pituitary tissue [34]. Ribosomal reference genes are often used in gene expression studies, but it was recently shown that ribosomal proteins are not stably expressed in pituitary adenomas [20], and thus the use of these genes as reference may lead to problems when interpreting the results. Also, ribosomal genes are often co-regulated, and GeNorm analysis does not correct for this [29]. Here, we show that the

expression of reference genes not only differs between species, but also between healthy and diseased tissue within one species. The commonly used reference gene gapdh was among the most stably expressed in the human samples, but not in murine and canine samples. Again it is possible that the ranking in reference gene expression stability is influenced by experimental conditions as previously described [35]. Interestingly, Optiz et al showed that all technical variation (RIN value ranges), differences in amount of RNA isolated (Nanodrop) or the possible variations due to cDNA synthesis (oligo-dT primers or random hexamers) are largely outweighed by the variations between biological samples [36]. Together, this emphasizes the need to analyze which reference genes should be used in each specific species and sample group.

Although GeNorm and Normfinder use different analytical approaches, the results were similar in all groups, as shown in Tables 2 and 3. GeNorm calculates the gene expression stability (M) of one gene based on the average pair-wise variation between all the studied reference genes [28]. Normfinder uses a model-based approach by which the overall variation in expression within sample groups of interest (intra-group variation) and the variation across the sample population groups (inter-group variation) are calculated for each evaluated reference gene. The combination of the two parameters of variation results in a stability value, which represents a practical measure of the systematic error that will be introduced when using a particular reference gene. The advantage of GeNorm is that it allows rapid selection of the optimal number of reference genes, which is not possible with Normfinder. In the present study, the six included reference genes were not sufficient to reach the cut-off value of 0.15 in the GeNorm analysis for human and murine pituitary tumor samples or combined healthy and tumor samples. However, it is difficult to use more than six reference genes in practice, especially if there is relatively little tissue available, as is often the case with samples obtained during pituitary surgery. Analysis of pairwise variation showed that two or three reference genes were sufficient for reliable normalization in the normal pituitary samples from humans and mice, and three or four reference genes were sufficient for the normal pituitary samples and the combined normal/tumor samples from dogs.

The human tumor samples came from different tumor types, which might have increased variation in gene expression between samples. Also, the number of human tissue samples is smaller than for mice and dogs, possibly adding to the variation in results. The variation in the murine tumor samples was surprising because genetically inbred mouse strains were used and we would have expected less genetic variation [25]. The expression of the reference genes in pituitary tissue samples was far more stable than that in canine pituitary side population cells, where the inclusion of five reference genes did not result in the required $M < 0.15$ [31]. Since the amount of RNA isolated from the pituitary side population (around 3% of total cell volume of a pituitary gland) was low, an amplification

step was necessary to acquire sufficient RNA and cDNA for expression studies, and it is possible that gene-specific amplification caused this apparent instability of gene expression. An alternative explanation for the lack of reference gene stability in canine pituitary side cells is that the composition of the pituitary side cell population is more variable than that of the pituitary gland as a whole.

In conclusion, the stability of expression of reference genes differs between species and between healthy/tumor tissue within one species. Quantitative analysis based only one reference gene, assumed to be stably expressed, might lead to wrong conclusions. This cross-species analysis clearly emphasizes the necessity to evaluate reference gene expression stability as a standard and integral part of proper experimental design and subsequent data analysis, in order to improve the validity of the conclusions drawn on quantitative molecular analyses.

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Disclosures

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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8 / Identification and characterisation of side population cells in the canine pituitary gland

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Abstract

To date, stem/progenitor cells have not been identified in the canine pituitary gland. Cells that efficiently exclude the vital dye Hoechst 33342 can be visualised and identified using fluorescence activated cell sorting (FACS) as a 'side population' (SP), distinct from the main population (MP). Such SPs have been identified in several tissues and display stem/progenitor cell characteristics. In this study, a small SP (1.3%, n = 6) was detected in the anterior pituitary glands of healthy dogs. Quantitative PCR indicated significantly higher expression of CD34 and Thy1 in this SP, but no differences in the expression of CD133, Bmi-1, Axin2 or Shh. Pro-opiomelanocortin (POMC) and Lhx3 expression were significantly higher in the MP than in the SP, but no differences in the expression of Tpit, GH or PRL were found. The study demonstrated the existence of an SP of cells in the normal canine pituitary gland, encompassing cells with stem cell characteristics and without POMC expression.

Keywords: Dog, Pituitary gland, Stem cell, Progenitor, Side population

Introduction

The anterior pituitary gland is central to the regulation of homeostasis, metabolism, growth, and reproduction [1] and contains five distinct cell types (corticotrophs, thyrotrophs, gonadotrophs, somatotrophs, and lactotrophs) that produce hormones in response to changing physiological and pathological conditions [2]. This may require the rapid expansion of one secretory cell type: for example, the population of lactotrophs expands substantially during pregnancy and lactation [3,4]. Under normal conditions, the mitotic rate in the pituitary gland is stable, with new cells being formed by the proliferation of differentiated cells, trans-differentiation of cells and maturation of undifferentiated cells [5,6].

It has been suggested that stem cells are key to the rapid expansion of cell populations [3-7]. Although a major limitation to the identification of stem cells is the paucity of specific markers, several are considered prospective identifiers, including CD34, CD133, Oct-4, Bmi-1 and Thy1 [3,4,8-10]. CD34 is a membrane antigen and haematopoietic stem cell (HSC) marker, CD133 is a membrane glycoprotein associated with the stem cell phenotype, although its function remains unclear, Bmi-1 is part of the polycomb repressive complex and plays a crucial role in self-renewal of haematopoietic and neural stem cells, Thy1 is a cell surface marker associated with spermatogonial or hepatic stem cells [3,4,8-10], and the transcription factor Oct-4 plays an essential role in the self-renewal of embryonic stem cells [11].

Furthermore, several well-conserved signalling pathways such as Wnt and Shh are pivotal in stem cell regulation and components of these signalling systems could also be indicative of a stem cell phenotype [3,4,12]. These pathways are also implicated in the embryonic development of the pituitary gland. More specific to the pituitary, the LIM homeobox transcription factors Lhx3 and Lhx4 regulate the development of the anterior pituitary: Lhx4 is down-regulated after pituitary development, whereas Lhx3 is not [13].

Since it is not possible to isolate stem or progenitor cells directly using specific markers, other methods are needed to isolate these cell populations. A method that has proven successful to identify and isolate stem and progenitor cells from multiple tissues is based on the 'self-defence' response of the cells to exclude toxins [14-17]. During these responses stem/progenitor cells expel a range of vital dyes, such as Hoechst 33342, and can be visualised by fluorescence activated cell sorting (FACS) as a side population (SP), forming a small 'streak' distinct from the main population (MP) [14-17]. This SP phenotype is mediated by membrane efflux pumps of the ATP-binding cassette transporter family [4,15-17].

The activity of these pumps is inhibited by verapamil, which results in the disappearance of the SP streak from the FACS plot [14-17]. Such an SP was first identified in murine bone marrow where it was enriched with HSCs [17]. Since then, SPs have been detected in numerous normal tissues, cell lines, and in tumours [15-17]. Chen et al. (2005) demonstrated that the murine anterior pituitary gland also contained a SP that displayed stem/progenitor cell and early embryonic characteristics [4].

The aims of the present study were to identify an SP in the anterior pituitary gland of healthy dogs and to characterise the expression of selected genes in this cell fraction by quantitative PCR (qPCR).

Materials and methods

Tissue sampling

Pituitary glands were obtained from six healthy, adult, crossbreed dogs euthanized in other experiments approved by The Ethics Committee on Animal Experimentation of The Faculty of Veterinary Medicine, Utrecht University, The Netherlands (DEC Number 2008.III.04.039). Immediately following euthanasia, the brain was removed and the pituitary gland detached and placed in a Petri-dish containing sterilised D-MEM (4500 mg/ L glucose containing L-glutamine and pyruvate) (GIBCO, Invitrogen) enriched with 1% penicillin streptomycin (GIBCO), 25 mM HEPES (Sigma–Aldrich), and 3% bovine serum albumin (BSA, Sigma–Aldrich). The anterior pituitary was carefully separated from the neurointermediate lobe and placed in a tube containing enriched D-MEM.

Cell dispersion

A modification of the pituitary cell dispersion protocol of Denef et al. (1989)[18] was used. Each anterior pituitary sample was dissected into small blocks using two sterile no. 10 blades and then transferred, in 3 mL of enriched D-MEM, into a 50 mL flask. The medium was replaced with 2 mL of 2.5 w/v% trypsin solution (GIBCO), and the tissue blocks were incubated for 18 min at 37 °C. Then 2 mL enriched D-MEM containing 4 µg of DNase (Sigma–Aldrich) were added for 1 min, to prevent the tissue blocks becoming coated by nucleohistone material released from damaged cells (Denef et al., 1989). The medium was removed and 2 mL of EDTA solution were added (HBSS, GIBCO/PAA), enriched with 1% penicillin streptomycin (GIBCO), 20 mM HEPES, 0.3% BSA and 2.5 mM EDTA (Sigma–Aldrich). After 5 min incubation at 37 °C, 2 mL of the enriched HBSS without EDTA were added and the tissue blocks were incubated for a further 15 min.

After three washes with 5 mL enriched HBSS, the tissue clumps were transferred to a Wheaton potter and carefully dispersed using the 'loose' followed by the 'tight' pestle. The cell suspension was then transferred to a 50 mL tube containing 5 µg DNase in 4.5 mL enriched D-MEM and filtered through a 70 µm nylon mesh filter (BD Biosciences). The cells obtained were centrifuged for 10 min at 190 g through a layer of 3 mL enriched D-MEM (3% BSA), and the pellet was re-suspended in serum-free chemically defined medium [28]. The cell suspension was then aspirated through a 0.9 mm needle, to disperse any remaining clumps and the cells were counted using a Burger-Türk cell counter.

Fluorescence activated cell sorting

The protocol of Chen et al. [4] was used and the cell density adjusted to 1 x 10^6 cells/mL. Control samples were created by incubating a 0.5 mL cell suspension with 100 µM verapamil (Sigma–Aldrich) for 20 min at 37 °C. All samples were then incubated with 2.5 µg/mL Hoechst 33342 (Sigma–Aldrich) for 90 min at 37 °C. Next, the cell suspensions were placed on ice to halt the efflux process and were centrifuged for 10 min at 190 g at 4 °C. The cell pellets were re-suspended in ice-cold PBS (GIBCO) with 2% fetal calf serum and 2 µg/mL propidium iodide (Sigma–Aldrich) prior to FACS analysis and sorting.

Flow cytometry was carried out on a FACS Vantage (BD Biosciences). The SP was visualised by dual-wavelength flow cytometry with ultra-violet excitation, using BP 424/44 (blue emission) and BP 630/22 (red emission) filters. The cell population was resolved into Hoechst^{low} SP and a Hoechst$^{mid+high}$ MP sub-populations by FACS which were then collected separately in 900 µL of extraction buffer (PicoPure RNA Isolation Kit, Arcturus Molecular Devices) [23]. The extraction buffer plus cells was heated for 30 min at 42 °C and centrifuged at 3000 g for 2 min. The supernatant was stored at -70 °C for further processing.

Gene expression analysis

RNA was isolated from the SP and MP samples using the PicoPure RNA Isolation Kit (Arcturus), according to the manufacturer's guidelines. RNA quantity and quality was measured with an Agilent BioAnalyzer 2100 (Agilent Technologies), and an RNA integrity number (RIN) was calculated by the analyser software. Typically an RIN ranges from '1' for highly degraded RNA, to '10' for highly intact RNA [19]. The RIN values of the RNA isolated in this study were between 8.1 and 10, indicating that the RNA quality was sufficient for quantitative PCR (qPCR). RNA was amplified using the RiboAmp Kit (Arcturus) and cDNA was synthesised with the iScript cDNA Synthesis Kit (Bio-Rad), both according to the manufacturers' instructions. A total of 100 ng of RNA was reverse transcribed in a volume of 20 µL. qPCR was performed in duplicate on 96 well iCycler iQ plates (Bio-Rad) in a spectrofluorometric thermal cycler (MyIQ, Bio-Rad). For each sample, a total volume of 25

µL was used, containing 0.2 µL cDNA, 13 µL iQ SYBR Green SuperMix (Bio-Rad), and 11.7 pmol of both forward and reverse primers. The primers used (Eurogentec S.A.; Table 1) were tested with a temperature gradient to determine the optimal annealing temperature (Ta) of the primer pair, and the specificity of the amplicon was confirmed by sequencing (data not shown). b-2-Microglobulin (b2M), the ribosomal protein L8 (RPL8), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the ribosomal proteins S5 (RPS5) and S19 (RPS19) were used as reference genes to normalise gene expression [20].

Reactions with a Ta < 58 °C started with 5 min at 95 °C, followed by 40 or 45 cycles of 20 s at 95 °C, 30 s at Ta, and 30 s at 72 °C. This reaction was continued for 30 s at 60 °C, followed by a melting curve, whereby the temperature was increased by 0.5 °C every 15 s over the temperature range 60–95 °C. When Ta > 58 °C, the incubation at 72 °C was omitted from each cycle [20]. There was no evidence of contamination on examination of 'no-template' or 'minus-RT' controls.

Statistical analysis

GeNorm software was used to evaluate reference gene stability [21] and statistical analysis was performed with R software (Version 2.12.0, Free Software Foundation). Expression of target genes in the SP and MP was normalized for the reference genes. A Kaplan–Meier survival analysis was performed to assess differences between SP and MP, after which the two groups were compared using a logrank test. Differences were considered significant where $P < 0.05$.

Table 1. Nucleotide sequences and annealing temperatures (Ta) of primers used in the quantitative PCR analysis.

Gene	NCBI Ref. seq.[a]	F/R	Sequence	Ta (°C)
CD34	NM_001003341	F	5'-TCAGGGCCCCCGACATCTC-3'	65.7
		R	5'-TCTCTGCTCACCCCTCTGGAAAAA-3'	
Thy1	XM_546483	F	5'-CAGCATGACCCGGGAGAAAAAG-3'	63.5
		R	5'-TGGTGGTGAAGCCGGATAAGTAGA-3'	
CD133	XM_545934	F	5'-CTGGGGCTGCTCTTTGTGAT-3'	60.4
		R	5'-AGGCCCCATTTTTCTTCTGTC-3'	
Bmi-1	XM_544225	F	5'-TGGACTGACAAATGCTGGAGAACT-3'	68
		R	5'-AGGGAACTGAGGATGAGGAGACTG-3'	
Axin2	XM_548025	F	5'-GAGACAAATGCGTGGATACCT-3'	60
		R	5'-TGCTTGGAGACAATGCTGTT-3'	
Shh	XM_845357	F	5'-GGGACGAGGACGGTCAC-3'	60
		R	5'-CACTGGCAGGAGCAGGG-3'	
Tpit	NM_001005758	F	5'-CATAGCTGTGACTGCCTAATC-3'	59
		R	5'-ACATGCTGGCTCTCAGAGAC-3'	
POMC	XM_844370	F	5'-GCCTGCAAGCCCGACCTCTC-3'	62
		R	5'-CTCCGCCCGCCGCCACCTCTTCTT-3'	
GH	NM_001008275	F	5'-CTGCTGCTCATCCAGTCGT-3'	60
		R	5'-CAGGTCCTTGAGCTTCTCGT-3'	
PRL	NM_001008275	F	5'-GAAGACAAGGAGCAAGC-3'	60
		R	5'-TGTGACTAGATGATACAGGG-3'	
Oct4	XM_538830	F	5'-ACCCTAGGAATATACTCAGGCG-3'	60.9
		R	5'-ACGGCAGATGGTGTTTGG-3'	
Lhx3	XM_844322	F	5'-ACTTCTTCAAGCGATTCGGGA-3'	68.1
		R	5'-GTAGAACTCGTCGCCTGTGG-3'	
B2M	XM_535458	F	5'-TCCTCATCCTCCTCGCT-3'	61.2
		R	5'-TTCTCTGCTGGGTGTCG-3'	
RPL8	XM_532360	F	5'-CCATGAAT/CCTGTGGAGC-3'	55
		R	5'-GTAGAGGGTTTGCCGATG-3'	
GAPDH	NM_001003142	F	5'-TGTCCCCACCCCCAATGTATC-3'	58
		R	5'-CTCCGATGCCTGCTTCACTACCTT-3'	
RPS5	XM_533568	F	5'-TCACTGGTGAG/AACCCCCT-3'	62.5
		R	5'-CCTGATTCACACGGCGTAG-3'	
RPS19	XM_533657	F	5'-CCTTCCTCAAAAAAGTCTGGG-3'	61
		R	5'-GTTCTCATCGTAGGGAGCAAG-3'	

F, forward primer; R, reverse primer; POMC, pro-opiomelamocortin; GH, growth hormone; PRL, prolactin; GAPDH, glyceraldehyde-3- phosphate dehydrogenase. [a] NCBI mRNA reference sequence.

Results

Fluorescence activated cell sorting

FACS analysis identified a Hoechst^{low} SP that comprised a mean of 1.3% (±0.73%, SD; n = 6) of the total cell population (Fig. 1A). The SP phenotype was confirmed by the disappearance of the Hoechst^{low} phenotype when verapamil was added during incubation with the dye. Pre-incubation with verapamil reduced the SP by 90.3 ± 10.0% (Fig. 1B).

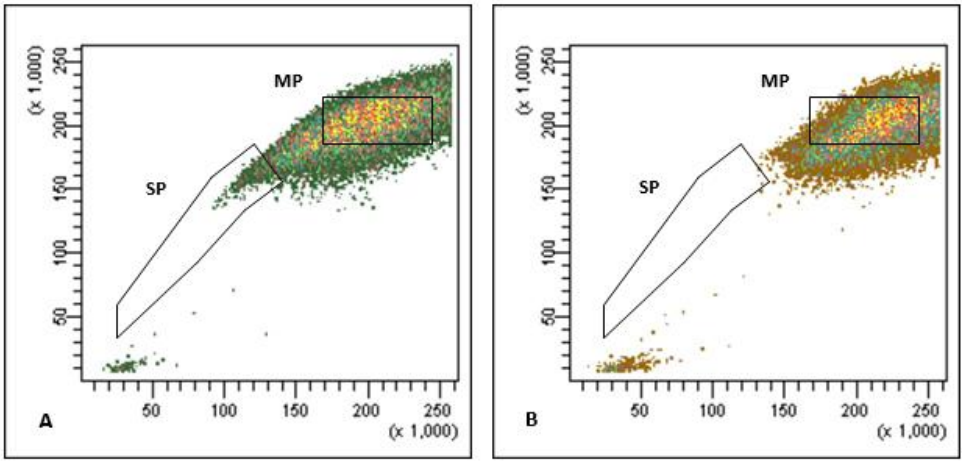

Figure 1. Fluorescence activated cell sorting analysis of canine anterior pituitary tissue after incubation with Hoechst 33342 dye. The side population (SP) comprises 1.3% of the total population cells (A). Hoechst dye efflux is significantly reduced in the presence of verapamil (B).

Gene expression analysis

Average reference gene expression in the SP and MP populations, increased for the following genes in the sequence: B2M; RPL8 and RPS19; GAPDH and RPS5. M-values decreased from 1.3 to 0.9 (Fig. 2A). Analysis of the optimal number of reference genes, indicated that the combination of these five genes gave values approaching 0.23 (Fig. 2B).

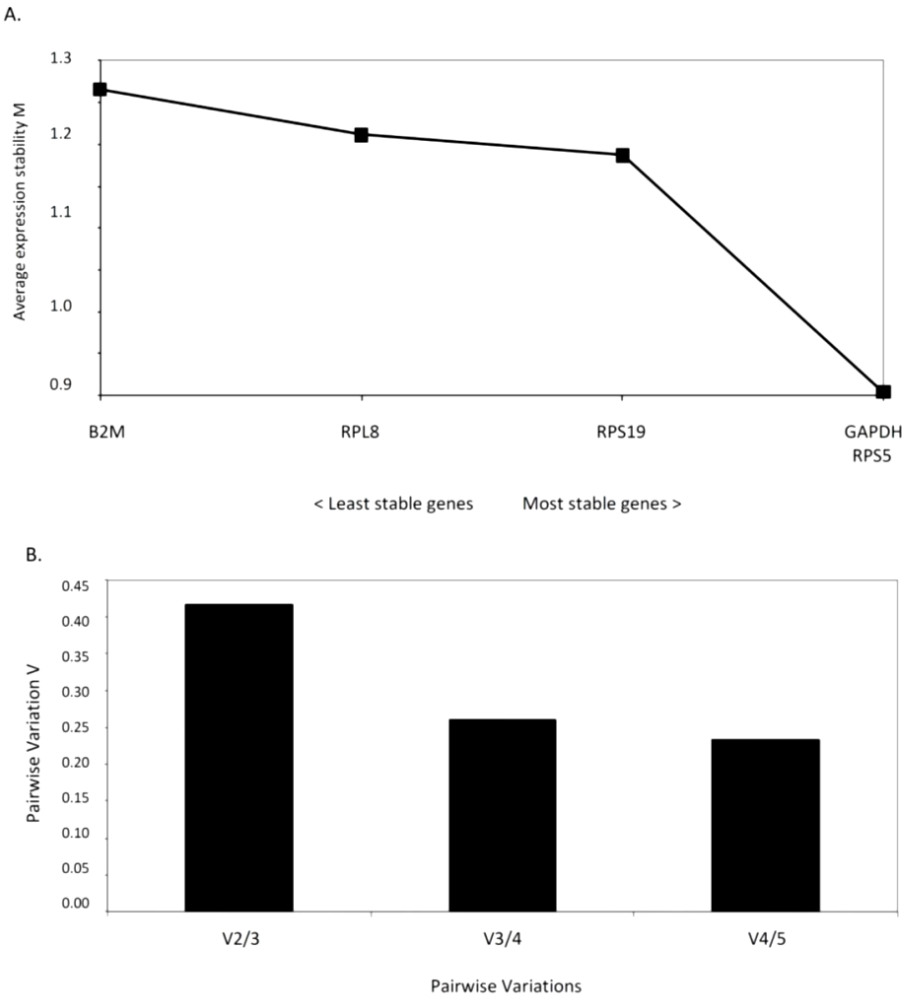

Figure 2. (A) Average expression stability values of five reference genes (B2M, RPL8, RPS19, GPDH, and RPS5). The geNorm program (see: http://medgen.ugent.be/~jvdesomp/genorm) calculates the gene expression stability (M) of one gene based on the average pair-wise variation between all the studied reference genes. The highest M values represent genes with the least stable expression, suggesting a less than optimal reference gene candidate. Step-by-step elimination of the least stable genes generates a ranking of reference genes according to their M values and ultimately results in the identification of the most stable genes [27]. (B) Determination of the optimal number of reference genes for normalisation. The geNorm program (see above) calculates the normalisation factor by assessing the optimal number of reference genes for generating the M factor by calculating the pair-wise variation V. The pair-wise variation between these genes defines V [27]. The lower V, the less the variation. V3/4 indicates the variation in normalisation factor with 3 vs. 4 reference genes.

SP and MP cells were sorted by FACS for qPCR expression analysis. Starting from 3.5 to 5.0 X106 anterior pituitary cells prior to incubation with Hoechst 33342, the number of cells sorted ranged from 4500 to 16,000 for the SP and from 50,000–61,000 for the MP. The amount of RNA isolated ranged from 2.7 to 6.0 ng for the SP and from 34.2 to 371.2 ng for the MP samples, respectively. Expression of genes generally recognised as markers of: stem/progenitor cells (i.e. CD34, CD133, Bmi-1, Thy1 and Oct-4); embryonic signaling pathways (Axin2 and Shh); pituitary development and hormonal lineages (Lhx3, Tpit, POMC, GH, and PRL): was compared between the SP and MP from each pituitary (Fig. 3). The expression of CD34 (Fig. 3A) and Thy1 (Fig. 3C) was significantly higher in the SP (P = 0.03 for CD34 and P = 0.002 for Thy1).

Expression of CD133 (Fig. 3B) and Bmi-1 (Fig. 3D) was not significantly different between the two cell populations and the embryonic marker Oct-4 was not detectable in either population. The expression of Axin2 (a specific target gene of the Wnt pathway; Fig. 3E) and of Shh (Fig. 3F) was not significantly different between the SP and MP. The LIM homeobox transcription factor Lhx3 (Fig. 3G) had a significantly higher expression in the MP (P = 0.01). The expression of pro-opiomelanocortin (POMC; Fig. 3H), the hormone precursor of ACTH, was significantly higher in the MP than in the SP (P = 0.02). The expression of Tpit (Fig. 3I), GH (Fig. 3J), and of PRL (Fig. 3K) was not significantly different between the two cell populations assessed.

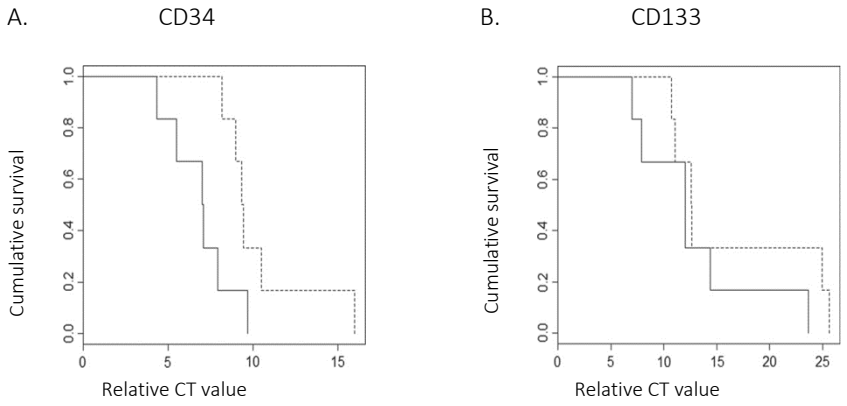

Figure 3. (continued on next page)

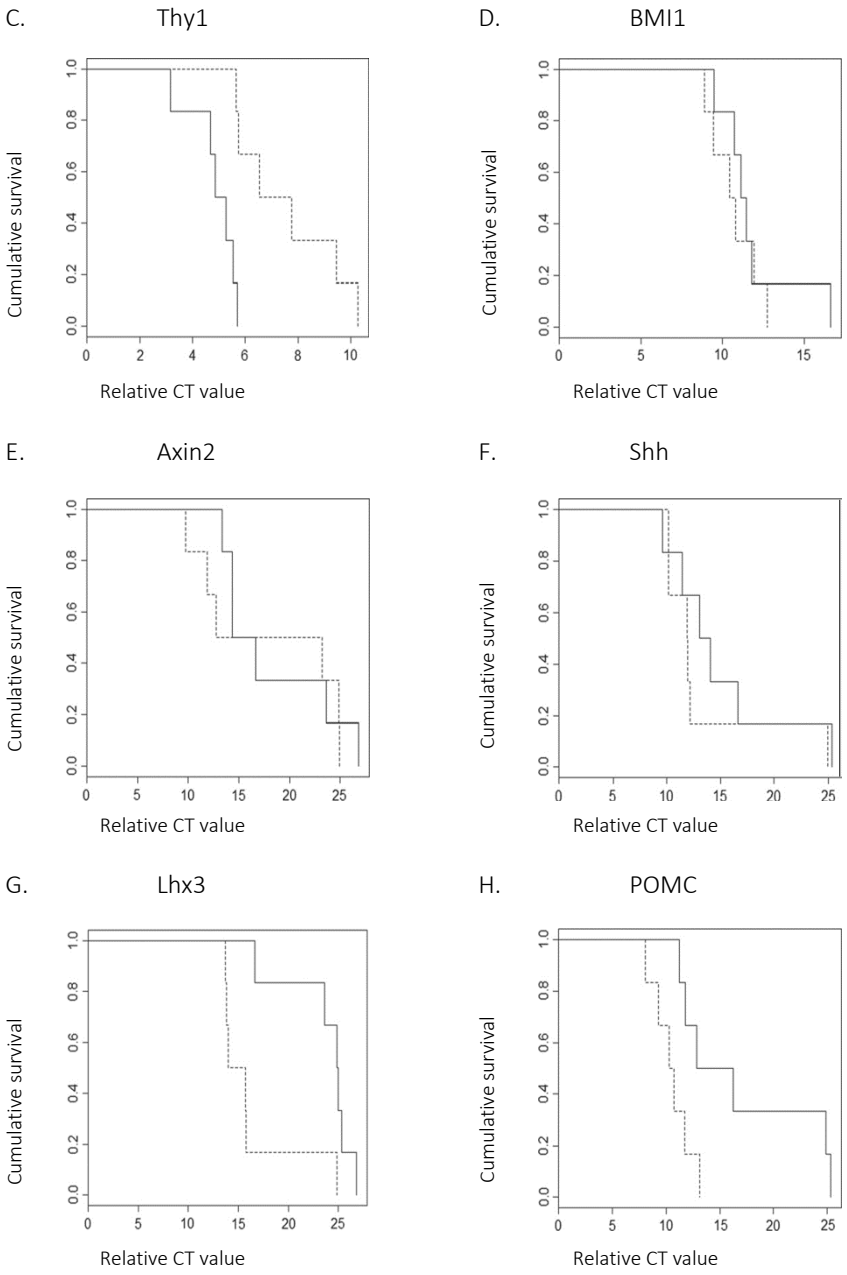

Figure 3. (continued on next page)

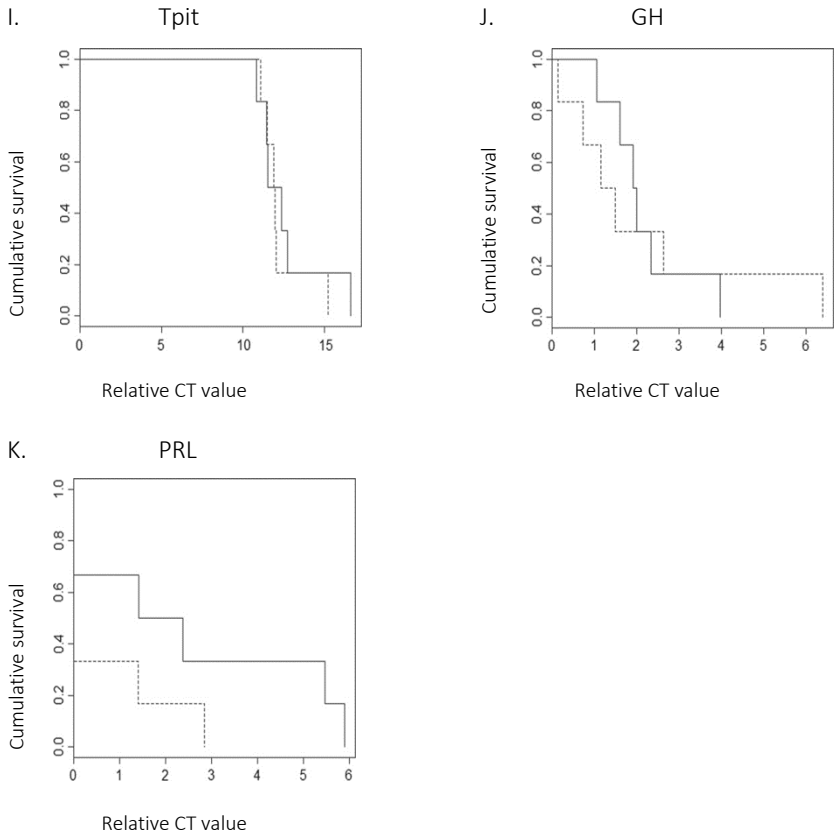

Figure 3. Kaplan–Meier curves (A–K), illustrating the probability of the expression of the target gene, expressed as a value between 0 and 1. A 'step-decrease' in the plot indicates expression of the gene in a sample at the given relative cycle threshold (CT) value. A lower plot indicates expression at lower CT values, i.e., greater expression. Continuous and dotted lines represent samples of SP and MP, respectively. Significant differences were found for the CD34, Thy1, POMC and Lhx3 genes ($P < 0.05$).

Discussion

FACS analysis of the SP phenotype is an effective method of isolating potential stem and progenitor cells within tissues [4,15]. This study has identified a SP of cells in the canine anterior pituitary for the first time. This phenotype was confirmed by the disappearance of the Hoechst^{low} cell fraction when the ABC-pump blocker, verapamil, was added. This cell phenotype has previously been demonstrated in the murine pituitary gland and in the canine liver [4,22,23].

As in the mouse, the SP identified in the canine pituitary represents a small fraction of the total cell population and contains cells expressing stem/progenitor cell markers [4]. We found a high level of expression of markers such as CD34 and Thy1 in the canine pituitary SP fraction. Markers of adult endocrine cells such as Lhx3 and POMC, were predominantly detected in the MP. These findings suggest that the SP is depleted from differentiated anterior pituitary corticotroph cells and encompasses cells with some characteristics of stem/progenitor cell phenotype. However other cells, such as those labelled with the CD133 and Bmi-1 markers, did not seem to exhibit an SP-related efflux capacity. It is noteworthy that the canine pituitary SP would be expected to represent a heterogeneous population, as found in other tissues, which might potentially mask the differential expression of some stem/progenitor cell-associated genes. This may also explain the varying gene expression found in the different samples.

The murine pituitary SP was also found to be heterogeneous in nature and significant further identification procedures were required to pin-point the constituent stem/progenitor cells [22]. In a similar way, upregulation of the CD34 marker may also signify the presence of HSC, or of endothelial progenitors, in the SP. The latter were also identified in the murine SP and were hypothesised to be involved in the remodelling of the capillary network during the period of pituitary plasticity [22].

Furthermore, inconsistencies in expression between the different samples may reflect an insufficiently stringent FACS-gating strategy which could have resulted in contamination of SP with MP, and MP with SP, cells, respectively. Similarly, the RNA amplification step possibly distorted some of the mRNA levels found. Given these potential confounding factors, further studies will be required to more unambiguously identify this SP.

In contrast to the murine pituitary SP, Oct-4 could not be detected in either the canine pituitary SP or MP. Signalling via Wnt appears to be active in the SP since its principal target gene, Axin2, was detected. This signalling pathway, emanating from the ventral diencephalon, is central to both adult stem cell renewal and to cell 'fate determination' in pituitary embryogenesis [4,12,22,24]. The Shh pathway plays an important role in both

the developing and fully mature pituitary gland, as well as in other endocrine tissues [24,25]. Whether or not Wnt and Shh signalling are generally more active in the SP than the MP remains unclear since both Axin2 and Shh exhibited greater expression in only half of the SP samples in the current study and were also upregulated in some of the MP samples. In the murine pituitary gland, Wnt and Shh pathway components were found to be upregulated in the SP, supporting the existence of stem/progenitor cells which either maintain their embryonic nature, or recapitulate to embryonic form [4,22].

The greatest expression of LIM homeobox transcription factor Lhx3 [13] was in the MP, consistent with its function in mature endocrine cells. In the murine pituitary, Lhx3 expression is also highest in the MP [22]. Chen et al. also demonstrated that expression of Lhx4 was highest in the SP subset of stem/progenitor cells [22]. Although Lhx4 is a further LIM homeobox transcription factor essential to pituitary embryogenesis that is, in contrast to Lhx3, strongly downregulated [13], analysis of its expression was not successful in the current study. Chen et al. also found that the hormone markers POMC, PRL, LH, FSH, TSH and GH, were expressed at higher levels in the MP than the SP [4]. In the present study, significantly higher expression of POMC was only found in the MP, and no significant differences in the expression of the other genes assessed, were found.

Although five reference genes, representing three independent biochemical pathways, were included in our study, the stability of gene expression was >0.15, the preferred upper level for reference gene stability [26]. In consequence, the pattern of gene expression might change slightly if it was corrected for these reference genes, with the desired stability, although this would not be likely to radically change our findings. Of greater importance is the fact that this suggests the use of only one or two reference genes is inadequate. Further studies are required to identify specific reference genes for the canine pituitary that fulfil Minimum Information for Publication of Quantitative RT-PCR Experiments (MIQE) criteria [26].

Conclusions

In this study we identified a SP of cells in the canine pituitary gland and gene expression analysis suggests this population contains cells of a stem/progenitor phenotype. However, given the fact that expression differences were not always consistent in our samples, further studies will be required to characterise this SP in more depth.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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9/ Summarizing discussion and conclusions

The pituitary gland is a small endocrine gland located at the base of the brain. It is crucial for the maintenance of several homeostatic functions, including metabolism, growth, and reproduction [1]. Although pituitary adenomas are quite regularly encountered in humans, the exact pathogenesis remains unclear [2]. Pituitary-dependent hypercortisolism (PDH) is a common endocrinopathy in dogs, with an estimated incidence of 1 or 2 in 1000 dogs/year [3]. It is caused by an adrenocorticotropic hormone (ACTH) secreting adenoma in the anterior or intermediate lobe of the pituitary gland. The clinical signs are caused by hypercortisolism, and in a small number of affected dogs also by the mass effect of the tumor [4]. Dogs with PDH can be treated with medication, radiation therapy or surgery. Surgical removal of the pituitary gland with transsphenoidal hypophysectomy has been used as treatment in the Netherlands since 1993 [5,6]. Although postoperative results are good, long-term recurrences do occur in around 25% of the dogs. Therefore, research focuses on pituitary tumorigenesis and aims to identify possible prognosticators and therapeutic targets to improve long-term results.

This thesis starts with a further introduction into pituitary morphology and development, clinical signs, diagnosis and treatment of dogs with PDH and Cushing's disease in humans. Subsequently, an overview of the current knowledge of pituitary tumorigenesis is given (Chapter 2). The aim of the first part (Chapter 3 and 4) was to analyze the long-term follow up of dogs with PDH treated with transsphenoidal hypophysectomy over a 20-year period and to study the prognostic value of peri-operative measurement of ACTH and cortisol in predicting recurrence of hypercortisolism. The second part of the thesis (Chapter 5, 6, and 7) focuses on possible prognostic pituitary tissue markers, studied with immunohistochemistry and gene expression analysis by means of quantitative PCR (qPCR) on pituitary tissue. In order to better understand pituitary tumorigenesis, the aim of Chapter 8 was to identify and characterize possible pituitary stem cells in the normal canine pituitary gland.

The long-term results in a cohort of 306 dogs treated over a 20-year period showed that transsphenoidal hypophysectomy is an effective treatment for dogs with PDH (Chapter 3). Remission was achieved in 92% of the dogs. The longest postoperative survival time in this group of dogs was more than 10 years, with a median survival time of 2 years. Earlier studies already showed that pituitary size, expressed as the pituitary height/brain area (P/B) ratio, was an important prognosticator [5,7]. Here, we not only showed that dogs with a higher P/B ratio have shorter survival and disease free periods, but we also found that the pituitary size of patients at time of surgery significantly increased over time. This increase is probably caused by the introduction of trilostane as a novel medical treatment for dogs with PDH during the same period [4]. Since trilostane treatment leads to comparable long-term results as surgical treatment [8], and has less side effects than mitotane treatment, patients where continued pituitary growth is not a major concern in

the short term (i.e. patients with a small pituitary adenoma), tend to be treated with trilostane medication first instead of surgery. This may explain a shift towards dogs with larger pituitary adenomas referred for pituitary surgery over the 20-year time span. Although dogs with larger tumors have a worse prognosis, the overall recurrence rate (27%) found in this study is comparable to previous reports [5,7]. Also, we found that although the P/B ratio of surgically treated patients significantly increased over the 20-year period, the recurrence rate did not change. This indicates an improved outcome in the subpopulation with larger tumors possibly due to both improved surgical experience with these large tumors and a better management of the postoperative complications. Based on these results, it would be interesting to further investigate the positive and negative predictive value of the P/B ratio, or a combination of the P/B ratio and pre-operative urinary cortisol to creatinine ratios (UCCRs). Although surgical treatment remains the favorable treatment for dogs with enlarged pituitaries, a subpopulation of these patients with negative pre-operative prognosticators might benefit from a personalized treatment with specific adjuvant therapies before or after surgery.

The recurrence rate of 27% indicates the need for surgical prognosticators in dogs treated with pituitary-dependent hypercortisolism. If patients with a higher risk of recurrence can be identified at an early time point before surgery or shortly after surgery, they can be monitored more closely, or they can be treated with adjuvant therapies, such as radiation therapy of the pituitary fossa [9-11]. In human medicine, stereotactic radiation therapy and recently proton therapy have been introduced in postoperative treatment of patients with pituitary adenomas [12,13]. Both treatments are effective, but recurrence of hypercortisolism does also occur after such treatments. Recurrences in dogs with PDH are thought to occur because of regrowth of the pituitary adenoma by microscopic islets tumor cells left behind during surgery or normal pituitary cells that transform into adenoma cells. Recently, a telescope and high definition camera system with 16x magnification has been used for better visualization during transsphenoidal hypophysectomy in dogs [14]. With this technique, the fossa can also be inspected for macroscopic pituitary tissue otherwise left behind. In human medicine, endoscopic pituitary surgery is commonly used [15] but anatomical differences between dogs and humans make this technique less applicable in canine surgery. However, there is definitely a place for endoscopy-assisted pituitary surgery in dogs, where the endoscope is used after removal of the pituitary tumor to check the fossa for pituitary remnants [16].

Because the short half-life of ACTH (around 20 minutes), a fast drop in plasma ACTH concentrations is expected after complete hypophysectomy [17] and plasma cortisol concentrations should follow this trend. Very low plasma ACTH concentrations immediately after surgery suggest complete removal of the corticotroph adenoma, but do not exclude presence of remaining normal pituitary corticotroph cells, because these cells

have been suppressed for a long period by the negative feedback of high cortisol levels. However, if functional corticotroph adenoma cells would have been left behind, it is likely that normal or elevated plasma ACTH concentrations would be measured immediately after surgery. Therefore it was hypothesized that immediate postoperative concentrations of ACTH and cortisol could be used as prognosticators for recurrences of PDH. In a cohort of 112 dogs, the prognostic value of peri-operative plasma ACTH and cortisol concentrations for recurrences was studied (Chapter 4). It was found that the evaluation of the individual peri-operative hormone curves of hypophysectomy patients provides valuable information about the risk of recurrence. However, it was not possible to identify an exact cut-off point to identify all recurrences with a single hormone measurement. In humans, it was reported that the combination of postoperative high plasma ACTH concentrations at the time of the lowest plasma cortisol concentrations identified all recurrences [18]. We did not find such a clear distinction between the groups of animals with and without recurrence. For the individual hypophysectomy case, the immediate postoperative ACTH profile remains a useful monitoring tool in the clinic to get a quick first impression within days after surgery to see whether treatment has been successful. However, looking at both dogs with and without recurrence, we encountered a large variation in peri-operative hormone concentrations.

Interestingly, we found that plasma cortisol concentrations are significantly different between dogs with and without recurrence, where plasma ACTH concentrations were not. In humans, postoperative cortisol is also widely used to study the risk for recurrence [19-21]. We hypothesize that, because cortisol secretion is a reflection of ACTH secretion, and the half-life of cortisol is longer than that of ACTH, the difference between the two groups is more pronounced and therefore statistically significant for cortisol and not for ACTH. Measuring plasma ACTH and cortisol concentrations for a longer time period after surgery and interpretation of the results is influenced by several factors such as desmopressin administration and the start of treatment with cortisone acetate after surgery. Desmopressin, a known ACTH-stimulating agent, was started at 1 hour after surgery, which may have stimulated (remnant) corticotroph cells and may have resulted in measurable plasma ACTH concentrations immediately after surgery. On the other hand the hypophysectomized patients need cortisone to prevent hypocortisolism, which interferes with cortisol measurement and inhibits ACTH secretion, if still present. In this study we did not prolong cortison deprivation after surgery for more than 5 hours to prevent side effects from severe hypocortisolism in the postoperative period. We can conclude that, although postoperative hormone concentrations can give additional information about the risk of recurrence, other clinical parameters remain essential, with the P/B ratio being the most important prognosticator.

Apparently, the larger the pituitary gland before surgery, the higher the risk of leaving cells behind. Possibly, not only remnant adenoma cells can cause recurrence, but also normal corticotroph cells that might remain after surgery can develop into an adenoma, because dogs show remission directly after surgery and develop recurrence of hypercortisolism after a period as long as four years. It is unclear why remaining cells develop into a functional adenoma (again) in time in some dogs. A possible explanation is a change in the direct cellular environment, as is described for liver metastases in human medicine [22]. It is also thought that hypothalamic factors contribute to development of pituitary adenomas [23,24], and these factors could also contribute to development of recurrence.

As clinical parameters are not enough to predict all recurrences and postoperative hormone concentrations vary among dogs with and without recurrence, we attempted to find prognosticators at the transcriptional level by the molecular approach of pituitary tumor tissue removed during surgical treatment. The pathogenesis of pituitary tumors is widely studied in mouse models. It has been shown that Rb +/- heterozygous mice develop pituitary adenomas, making them an interesting model [25]. Several mechanisms are thought to play a role in pituitary tumorigenesis (Chapter 2), including cell cycle disruption and disruption of signaling pathways that play a role in pituitary development [2,24]. Therefore, the second part of the thesis focused on the expression of possible prognostic markers in canine pituitary adenomas. Where in human literature proliferation markers are widely used as prognosticators [26], we found that the expression of the proliferation markers Ki-67 and proliferating nuclear antigen (PCNA) were not significantly different between enlarged and non-enlarged pituitaries (Chapter 5). Apparently, it is not an increased proliferation activity that leads to the difference between a non-enlarged and enlarged adenomas, but more a disruption of cell cycle regulation. The cell cycle inhibitor p27kip1 trended to be expressed less in enlarged pituitary glands compared to non-enlarged pituitary glands. p27kip1 normally drives pituitary progenitor cells out of cell cycle to differentiate into hormone producing cells [27]. In mouse models, disruption of the gene coding for p27kip1 caused intermediate lobe pituitary adenomas [28]. Further analysis of p27kip1 expression at both protein and gene levels, for example in dogs with and without recurrence might be an interesting future objective.

Around 10 to 15% of the canine corticotroph pituitary adenomas originate in the intermediate lobe. The outcome of dogs with an intermediate lobe adenoma is worse than that of dogs with an anterior lobe adenoma [7,29], indicating the possible usefulness of melanotropic markers as prognosticators or for the development of melanotroph specific targeted therapies [30]. Although it was shown that around 30% of the canine pituitary adenomas express the melanotroph specific transcription factor paired box protein 7 (Pax7) [30,31], we were unable to correlate Pax7 expression or activation of the

Pax7 signaling pathway to clinical parameters and outcome (Chapter 6). We found Pax7 expression in both adenomas originating in the anterior and intermediate lobe, but also found that multiple tumors were negative for Pax7. Although Pax7 is not useful as a direct prognostic marker, it remains an interesting target for further studies unraveling the tumorigenesis of pituitary adenomas. Possibly, the disruption of normal pituitary development leading to an adenoma occurs in cells that are not differentiated yet into a specific lineage, i.e. stem cells, which were further explored in Chapter 8.

Isolation of stem cells is hampered by the lack of tissue specific stem cells markers. A possible pituitary stem cell marker is sex determining region Y-box 2 (Sox2). Sox2 maintains pluripotent capacity of embryological stem cells, together with other transcription factors [32]. In the adult pituitary gland, a small population of cells maintains expression of Sox2, and these cells express several stem cell characteristics [33]. Also it was reported that $Sox2^{+}$ cells give rise to pituitary adenomas in transgenic mice [34]. Although Sox2 seems to be a marker for stem cells, we could not relate Sox2 expression to clinical parameters in canine pituitary corticotroph adenomas (Chapter 6). As such, Sox2 cannot be used as a prognostic factor, but a role in canine pituitary tumorigenesis cannot be excluded based on these findings. A possible explanation is the location of the pituitary stem cell population in a specific niche around the pituitary cleft [35,36]. With the analysis of expression in a undefined fragment of the pituitary adenoma or a healthy control, Sox2 activity between adenomas and normal pituitaries was not different. It would be interesting to study the expression of Sox2 in the specific stem cell niche in both healthy pituitary tissue and pituitary adenomas and to analyze if there is upregulation of Sox2 in specific areas, more clearly defining this stem cell niche in canine pituitary glands. Identification of a specific stem cell location may lead to easier isolation of these stem cells, for example by advanced techniques as microdissection.

Based on the qPCR results described in Chapter 8, we found that the reference genes most commonly used in canine studies were not stably expressed in canine pituitary tissue. Therefore, the expression of six frequently used reference genes was studied in pituitary tissue (normal and adenoma) from three species (humans, mice, and dogs) (Chapter 7). These species were chosen since they are the most studied species in pituitary research. We found that the stability of expression of the reference genes differed between species and between healthy and diseased tissue within one species. This emphasizes the need to evaluate the expression stability of reference genes as a standard and integral aspect of study design and data analysis.

The results acquired by the molecular approach of pituitary tumors suggests that the pathogenesis of pituitary adenomas in dogs might not be as similar to the pathogenesis in humans as shown previously for somatostatin and dopamine receptor expression [37].

Although canine corticotroph adenomas provide an interesting model to study Cushing's disease, differences between humans and dogs should be taken into account when using dogs as models of human disease [38]. Apparently, canine corticotroph adenomas differ from corticotroph adenomas in humans in some of their pathobiological characteristics as was not only shown by differences in expression of proliferation markers and transcription factors on protein level, but also by the differential expression of reference genes.

The possible role of stem cells in pituitary development and pituitary tumorigenesis was already mentioned in Chapter 6 but needs further investigation and this is the subject of Chapter 8 of this thesis. Because of the cellular plasticity of the pituitary gland, with rapid expansion of cell populations in response to homeostatic changes, it is thought that the adult pituitary contains stem cells [35,39-44]. It is shown that in the rat pituitary gland, approximately 30% of cells arise from mitosis of already differentiated cells, whereas the others are produced from undifferentiated cells or possible stem cells [45]. Isolation of stem cells is complicated by the lack of specific stem cell markers. A promising way to isolate potential stem cells is by means of fluorescence activated cell sorting (FACS) to sort a side population (SP) of cells, which contains stem cells in multiple tissue types [46]. The SP was first isolated in the murine pituitary gland by the research group of Vankelecom in Leuven, Belgium [40]. With use of their protocols, we successfully isolated and characterized a SP from six healthy canine pituitary glands (Chapter 8). We used qPCR analysis to study the expression of genes generally recognized as markers of: stem/progenitor cells (i.e. CD34, CD133, Bmi-1, Thy1, and Oct-4); embryonic signaling pathways (Axin2 and Shh); pituitary development and hormonal lineages (Lhx3, Tpit, POMC, GH, and PRL). Expression was compared between the SP and main population (MP) from each pituitary. A significantly higher expression of CD34 and Thy1 was found in the SP indicating stem cell characteristics of these cells. POMC and Lhx3 expression was significantly higher in the MP than in the SP, indicating that these cells are more differentiated cells. Although these results were promising, expression patterns were not consistent in all samples and the sample number was low. Therefore, we are currently investigating the presence of a side population in a larger sample number of both healthy canine pituitaries and pituitary adenomas.

In conclusion, transsphenoidal hypophysectomy is an effective treatment for dogs with PDH and provides material for continued research into the pathobiology of pituitary adenomas in dogs. It remains unclear why, after initial remission, some dogs develop recurrence of hypercortisolism, whereas others do not. Because the surgical technique is different from that in human medicine (total hypophysectomy in dogs versus adenomectomy in humans), the pathobiology of recurrence might differ between species. We found that in dogs, not every recurrence can be predicted with clinical parameters. Therefore, the search for molecular prognostic markers is continued. Of the markers

studied in this thesis, p27kip1 and Pax7 remain interesting for future studies, indicating a disruption of cell cycle control as the basis for pituitary tumorigenesis, but they cannot be used as direct prognostic markers. The role of stem cells in the pituitary gland and pituitary tumorigenesis remains a promising research field, but continued studies are needed to unravel the role of stem cells in canine pituitary adenomas. Since pituitary tissue of a patient is usually only available after surgery, the markers studied in this thesis can only be evaluated when the patient is already operated.

What is urgently needed is a pre-operative non-invasive specific biomarker to predict surgical outcome, i.e. remission or recurrence. The latest development in molecular profiling of serum derived tissue specific miRNA levels might offer such a useful set of biomarkers. This is one of the current lines of canine pituitary research in tPIT (the Pituitary Investigation Team).

Key Findings

Clinical parameters

- Transsphenoidal hypophysectomy is an effective treatment in dogs with PDH.
- The P/B ratio remains the most important clinical prognosticator for outcome of surgery in dogs with PDH.
- The size of pituitary tumors of dogs with PDH referred for pituitary surgery over the past 20 years has increased.
- Perioperative plasma ACTH and cortisol concentrations are a valuable addition as prognostic factors for outcome of surgery in dogs with PDH. However, there was no exact cut-off value to identify every recurrence after surgery.

Molecular parameters

- The expression of proliferation markers Ki-67 and PCNA is not different between enlarged and non-enlarged pituitary adenomas.
- The cell cycle inhibitor p27kip1 remains an interesting target for further studies.
- The expression of Pax7 and Sox2 in canine pituitary adenomas could not be related to clinical parameters.
- The expression of reference genes differs between pituitary tissue dogs, humans and mice.
- The pathobiological characteristics of canine pituitary adenomas seem to differ from human pituitary corticotroph tumors.
- The healthy canine pituitary gland contains a side population, displaying stem cell characteristics.

Future directions

- Isolation and characterization of a side population in a larger set of healthy pituitary glands and canine corticotroph adenomas.
- Study the role of stem cells in pituitary tumorigenesis.
- Development of a pre-operative non-invasive biomarker to predict surgical outcome.

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10/ Nederlandse samenvatting, curriculum vitae, list of publications, dankwoord

Nederlandse Samenvatting

De hypofyse is een kleine hormoonproducerende klier, die zich onderaan de hersenen bevindt. De hypofyse speelt een cruciale rol in het onderhoud van verschillende lichaamsfuncties, zoals metabolisme, groei en voortplanting. Wanneer er een (goedaardige) tumor in de hypofyse ontstaat, produceert deze een teveel aan het hormoon ACTH hetgeen leidt tot de ziekte van Cushing, ook wel hypofyse-afhankelijke hypercortisolisme genoemd. De ziekte van Cushing is een veel voorkomende endocriene ziekte bij de hond, met een geschat voorkomen van 1 of 2 op 1000 honden per jaar. De klinische verschijnselen zijn veel eten, veel drinken, veel plassen, een afwijkende vacht, een dikke buik en een verminderd uithoudingsvermogen. Een klein deel van de honden ontwikkelt ook neurologische verschijnselen ten gevolge van de tumor. Honden met de ziekte van Cushing kunnen behandeld worden met medicijnen, bestraling of een operatie. Het operatief verwijderen van de hypofyse met transsfenoïdale hypofysectomie wordt sinds 1993 als behandeling bij de hond toegepast in Nederland. Hoewel de resultaten na een dergelijke operatie goed zijn, komt recidief op de lange termijn voor bij ongeveer 25% van de honden. Daarom richt dit onderzoek zich op het ontstaansmechanisme van hypofysetumoren met als doel om mogelijke prognostische (voorspellende) factoren en therapeutische aangrijpingspunten te identificeren.

Dit proefschrift begint in ***Hoofdstuk 2*** met een verdere introductie over de morfologie en ontwikkeling van de hypofyse, de klinische verschijnselen, de diagnose en behandeling van honden met hypofyse-afhankelijke hypercortisolisme en de ziekte van Cushing bij de mens. Vervolgens wordt er een overzicht gegeven van de huidige kennis van tumorontwikkeling in de hypofyse. Het doel van het eerste deel van dit proefschrift (***Hoofdstuk 3 en 4***) is het analyseren van de lange termijn follow-up van honden met de ziekte van Cushing, die behandeld zijn met transsfenoïdale hypofysectomie in een periode van 20 jaar. Daarnaast wordt ook de prognostische waarde onderzocht van hormoonbepalingen van ACTH en cortisol rondom de operatie voor het voorspellen van recidief van hypercortisolisme. Het tweede deel (***Hoofdstuk 5, 6 en 7***) concentreert zich op mogelijke prognostische weefselmarkers in de hypofyse, waarbij gebruik wordt gemaakt van immunohistochemische kleuringen en gen expressie analyse met qPCR op hypofyse weefsel. Om de ontwikkeling van tumoren in de hypofyse beter te begrijpen, is het doel van ***Hoofdstuk 8*** om de stamcellen in hypofyses van normale honden te isoleren en te karakteriseren als basis voor toekomstig onderzoek aan de hypofyse tumor stamcel.

De lange termijn resultaten van 306 honden die gedurende een periode van 20 jaar zijn behandeld, laten zien dat transsfenoïdale hypofysectomie een effectieve behandeling is voor honden met de ziekte van Cushing (***Hoofdstuk 3***). In 92% van de honden is een remissie (normalisering van de hormoonwaarden) bereikt. De langste overlevingstijd na

deze operatie is meer dan 10 jaar, met een mediane overleving van 2 jaar na de operatie. Eerdere studies laten zien dat de grootte van de hypofyse, uitgedrukt als de ratio van de hypofyse hoogte/oppervlakte van de hersenen (P/B ratio) een belangrijke prognostische factor is. In ***Hoofdstuk 3*** laten we niet alleen zien dat honden met een hogere P/B ratio een kortere overlevingstijd en ziektevrije periode hebben na de operatie, maar ook dat de hypofyse-grootte van patiënten, op moment van de operatie in de loop van de tijd geleidelijk is toegenomen. Waarschijnlijk heeft deze toename in grootte te maken met de introductie in 2002 van de therapie met het medicijn Trilostane. Behandeling met Trilostane is gericht op vermindering van de hormoon productie uit de bijnierschors en gaat gepaard met minder bijwerkingen dan eerdere medicijnen (zoals Mitotane). Waarschijnlijk zijn daarna honden met een kleine hypofyse tumor eerder medicamenteus behandeld en honden met een grotere hypofyse tumor eerder voor chirurgie doorgestuurd. Hoewel honden met een grotere tumor een slechtere prognose hebben, is het gevonden aantal recidieven (27%) vergelijkbaar met eerdere studies. Blijkbaar is de uitkomst van de honden met een groter hypofyse tumor na chirurgie verbeterd, mogelijk door een combinatie van meer chirurgische ervaring en een betere behandeling van mogelijke complicaties die optreden na de operatie.

Aangezien het aantal recidieven op lange termijn 27% is, zijn prognostische factoren om de uitkomst na chirurgie te voorspellen wenselijk. Als patiënten met een hoger risico namelijk eerder herkend kunnen worden, dan kunnen zij postoperatief beter gecontroleerd worden of kunnen ze aanvullende behandelingen ondergaan.

Gedacht wordt dat een recidief ontstaat door hergroei van de tumor uit cellen die tijdens de operatie achterblijven. Bij een volledige verwijdering van de hypofyse tijdens de operatie verwachten we een snelle daling van de hormoon concentraties van ACTH en cortisol. Lage hormoonconcentraties kort na de operatie suggereren dat alle tumorcellen zijn verwijderd. Bij mensen worden hormoonconcentraties rondom hypofyse operaties gebruikt om de uitkomst voor patiënten te voorspellen. In ***Hoofdstuk 4*** wordt de prognostische waarde van ACTH en cortisol concentraties rondom hypofyse operaties bij 112 honden bestudeerd. Het blijkt dat de evaluatie van het verloop van de hormoonconcentraties rondom de operatie voor de individuele hond waardevolle informatie oplevert over het risico op een recidief. Het is echter niet mogelijk om een exacte grenswaarde te vinden waarbij alle toekomstige recidieven geïdentificeerd kunnen worden met een enkele hormoonbepaling. Bij mensen met de ziekte van Cushing is gevonden dat met een combinatie van een hoge concentratie ACTH in combinatie met een lage concentratie cortisol na een operatieve behandeling alle toekomstige recidieven kunnen worden geïdentificeerd. Wij vinden bij honden echter niet zo'n duidelijk onderscheid tussen de groepen dieren met en zonder recidief. Wel zijn cortisol concentraties tussen honden met en zonder recidief significant verschillend, maar is er

geen significant verschil in ACTH concentraties. Voor de individuele patiënt geeft het verloop van ACTH waarden gedurende de eerste dagen na de operatie een eerste, snelle indruk of de tumor in zijn geheel is verwijderd. Maar als we naar de grote groepen honden kijken zien we een grote variatie in hormoonwaarden rondom de operatie. Concluderend kan worden gesteld dat andere klinische parameters essentieel blijven, met de P/B ratio als belangrijkste prognostische factor. Blijkbaar is het zo dat hoe groter de tumor is, hoe groter het risico is dat er cellen achterblijven. Mogelijk kunnen niet alleen achtergebleven tumorcellen voor hergroei zorgen, maar ook normale hypofysecellen die zich op termijn tumoreus ontwikkelen. Er zijn namelijk honden die na de operatie eerst in remissie gaan en vervolgens pas een recidief krijgen na een periode van wel vier jaar na de operatie. Het is onduidelijk waarom achterblijvende cellen zich in sommige honden tot een functionele tumorcellen ontwikkelen. Mogelijk spelen veranderingen op celniveau daarbij een rol.

Omdat klinische parameters niet voldoende blijken om alle recidieven te voorspellen trachten we prognostische factoren te vinden op moleculair niveau, in hypofyseweefsel dat tijdens de operatie wordt verwijderd. Verschillende mechanismen op celniveau spelen een rol bij het ontstaan van hypofyse tumoren, onder andere door verstoring van de normale celdelingscyclus en verstoring van signaalpaden die een rol spelen in de ontwikkeling van de hypofyse. In het tweede deel van dit proefschrift (***Hoofdstuk 5 tot 8***) ligt de focus op de expressie van mogelijke prognostische markers in hypofyse tumoren bij de hond.

In onderzoek naar hypofysetumoren bij de mens wordt veel gebruik gemaakt van delingsmarkers als prognostische factoren. In ***Hoofdstuk 5*** laten we zien dat er geen significant verschil is in expressie van de delingsmarkers Ki-67 en PCNA in vergrote en niet vergrote hypofyse tumoren. Blijkbaar is het niet zozeer een verschil in delingsactiviteit dat zorgt voor het verschil tussen niet vergrote en vergrote tumoren, maar meer een verstoring in de regulering van de celdelingscyclus. Het gevonden verschil in expressie van de celcyclus-remmer p27kip1 neigt naar significant lagere waarde in vergrote hypofyses ten opzichte van niet vergrote hypofyses. Normaal gesproken zorgt p27kip1 ervoor dat hypofysecellen stoppen met delen en zich gaan specialiseren tot hormoonproducerende cellen. Daarom zou het interessant zijn om de activiteit van p27kip1 in de toekomst verder te onderzoeken op eiwit-niveau en op gen-niveau.

Ongeveer 10 tot 15% van de hypofysetumoren bij de hond ontstaat niet in de voorkwab maar in de tussenkwab (pars intermedia) van de hypofyse. Deze tumoren zijn meestal groter en honden met deze tumor hebben vaak een slechtere uitkomst na een operatie dan honden met een voorkwab tumor. Daarom wordt gedacht dat een marker specifiek voor middenkwab cellen (melanotrofe cellen) gebruikt kan worden als prognostische

marker of voor de ontwikkeling van specifiek gerichte therapieën. Hoewel ongeveer 30% van de hypofysetumoren bij de hond de melanotrofe transcriptiefactor Pax7 tot expressie brengt, kunnen we Pax7 expressie niet relateren aan klinische parameters of de uitkomst na operatie (***Hoofdstuk 6***). Hoewel Pax7 dus niet geschikt is als directe prognostische marker, blijft het wel een interessant doel voor verder onderzoek naar de ontstaanswijze van hypofyse tumoren.

Mogelijk ontwikkelt een hypofyse tumor zich niet uit de hormoonproducerende cellen in de klier, maar uit nog niet gedifferentieerde cellen, de zogenaamde stamcellen. Omdat er geen specifieke stamcel markers bestaan, is isolatie van deze stamcellen lastig. Een mogelijke stamcel marker voor de hypofyse is Sox2. In ***Hoofdstuk 6*** blijkt dat expressie van Sox2 in hypofyse tumoren bij de hond niet te relateren is aan klinische parameters. Een rol in het ontstaan van hypofyse tumoren is daarmee echter niet uit te sluiten.

Om expressie van mogelijke markers op gen niveau te onderzoeken wordt gebruikt gemaakt van qPCR studies. Hierbij worden de resultaten genormaliseerd met behulp van zogenaamde referentiegenen; dat zijn genen die in een bepaald weefsel stabiel tot expressie komen. Om deze referentiegenen vast te stellen voor hypofyseweefsel onderzoeken wij in ***Hoofdstuk 7*** de expressie van zes veel gebruikte referentiegenen in hypofyseweefsel (normaal en tumorweefsel) van mensen, muizen en honden. We vinden dat de expressie verschilt tussen gezond en ziek weefsel en tussen de verschillende diersoorten. Dit benadrukt de noodzaak om referentiegenen per onderzoek vast te stellen.

De mogelijke rol van stamcellen in de ontwikkeling van de hypofyse en het ontstaan van hypofysetumoren is het onderwerp van ***Hoofdstuk 8***. Een veelbelovende manier om stamcellen te isoleren is met behulp van “fluorescence activated cell sorting” (FACS) waarbij een zogenaamde side population (SP) van cellen wordt geïsoleerd. Deze SP blijkt stamcellen te bevatten in meerdere soorten weefsels, zo ook in de hondenhypofyse. In ***Hoofdstuk 8*** beschrijven we de succesvolle isolatie en karakterisering van de SP in 6 honden hypofyses. Met behulp van qPCR wordt de expressie van genen die een rol spelen in stamcellen, de embryonale signaalpaden, de hypofyse ontwikkeling en de hormoonproductie onderzocht. De SP blijkt een verhoogde expressie te hebben van enkele stamcelmarkers, terwijl de resterende cellen (de MP, main popluation) juist een verhoogde expressie heeft van markers voor de hormoonproductie van de meer ontwikkelde cellen. Deze resultaten zijn veelbelovend, maar de expressie is niet consistent in alle monsters en bovendien is het aantal monsters klein. Daarom wordt momenteel de SP in een groter aantal monsters onderzocht van zowel gezonde hypofyses als hypofyse tumoren.

Concluderend kan worden gesteld dat transsfenoïdale hypofysectomie een effectieve behandeling is voor honden met hypofyse-afhankelijke hypercortisolisme. Het blijft onduidelijk waarom sommige honden een recidief van hypercortisolisme ontwikkelen, terwijl dat bij andere honden niet het geval is. Omdat de operatie bij mensen anders wordt uitgevoerd dan bij de hond, kan de oorsprong en ontstaanswijze van de recidieven ook anders verlopen. Uit de resultaten van onze studies is dan ook gebleken dat de ontstaanswijze van hypofyse tumoren bij de hond mogelijk niet zo vergelijkbaar is met die van bij de mens als eerder wel werd gedacht. Hoewel de hond een interessant diermodel zou zijn om de ziekte van Cushing verder te bestuderen, moeten verschillen tussen hond en mens dus duidelijk in acht worden genomen.

We vinden dat niet elk recidief voorspeld kan worden met klinische parameters. Daarom wordt de zoektocht naar mogelijke moleculaire prognostische markers voortgezet. Van de markers die in dit proefschrift zijn onderzocht, zijn p27kip1 en Pax7 het meest interessant voor toekomstig onderzoek. Dit impliceert dat de basis van het ontstaan van hypofyse tumoren gezocht moet worden in een verstoring van regulering van de normale celcyclus. De rol van stamcellen blijft een veelbelovend onderzoeksveld, maar er is meer onderzoek nodig om de rol van stamcellen in de hypofyse en het ontstaan van hypofyse tumoren te ontrafelen.

Omdat hypofyse (tumor)weefsel van een patiënt alleen beschikbaar komt na een operatie, kunnen de weefsel markers die bestudeerd worden in dit proefschrift alleen worden onderzocht nadat de patiënt is geopereerd. Wat dringend nodig is, is een pre-operatieve, niet-invasieve specifieke marker die de uitkomst van de behandeling vooraf kan voorspellen (remissie of een recidief). Mogelijk kan het onderzoek naar weefselspecifieke miRNAs hierin een rol spelen. Dit is een van de huidige onderzoekslijnen binnen het hypofyse onderzoek.

Curriculum Vitae

The author of this thesis was born on November 11, 1984 in Groningen. She grew up in the small town of Haren. She attended high school in Groningen and after graduating in 2002, she spend six months in Costa Rica, where she worked as a volunteer in a National Park, before starting her study of Veterinary Medicine at Utrecht University in 2003. During her veterinary training, she participated in the honors minor at the faculty of humanities (2006-2008), the honors program at the faculty of veterinary medicine (2007-2008) and the leadership program for veterinary students at Cornell University, Ithaca, NY, USA (2009). In 2010, she won the basic science award of the Dutch Animal Cancer Fund (NKFD) for her research project on canine pituitary adenomas. She graduated cum laude in 2011 and subsequently started a surgical internship, which enabled her to continue her research project that led to this thesis. She currently works as a resident in small animal surgery (ECVS) at the Department of Clinical Sciences of Companion Animals, Utrecht University. In her free time, the author loves cycling and traveling, together with her husband Thijs.

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