Pathobiology and oncogenesis of pituitary corticotroph adenomas in dogs

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Pathobiology and oncogenesis of pituitary corticotroph adenomas in dogs

Pathobiologie en oncogenese van corticotrope hypofyse adenomen bij de hond

(met een samenvatting in het Nederlands)

Patobiologi och onkogenes för kortikotropa hypofysadenom hos hund

(med sammanfattning på svenska)

Proefschrift

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to Johannes, Simon and Jakob

CONTENTS

Abbreviations

ACTH	adrenocorticotropic hormone, adrenocorticotropin
α-MSH	α-melanocyte-stimulating hormone, melanotropin
α-GSU	α-glucoprotein subunit
AUC	area under the curve
AVP	arginine vasopressin
bHLH	basic helix-loop-helix
BMP	bone morphogenic protein
BrdU	bromodeoxyuridine
Brg1	brahma-related gene
cAMP	cyclic adenosine monophosphate
CDI	central diabetes insipidus
cdk	cyclin-dependent kinase
cDNA	copy DNA, a DNA copy of messenger RNA
CI	confidence interval
CLIP	corticotropin-like intermediate-lobe peptide
CPHD	combined pituitary hormone deficiency
CRH	corticotropin-releasing hormone
CT	computed tomography
CV	coefficient of variation
DA	dopamine
DNA	deoxyribonucleic acid
END	endorphin
ER	estrogen receptor
FGF	fibroblast growth factor
FSH	follicle-stimulating hormone
FSHβ	follicle-stimulating hormone beta subunit
GABA	gamma-aminobutyric acid
GADD45γ	growth arrest and DNA damage-inducible 45γ
GATA-2	GATA-binding protein 2
GH	growth hormone
GHRH	growth hormone-releasing hormone
GnRH	gonadotropin-releasing hormone
GSD	German Shepherd dog
HAH	hypofyse-afhankelijk hyperadrenocorticisme
HDAC2	histone deacetylase-2
HE	hematoxylin and eosin
HHA	Hypofysberoende hyperadrenokorticism
HPRT	hypoxanthine phosphoribosyltransferase
HR	hazard ratio

HRP	horseradish peroxidase
HX	hypophysectomy
ICU	intensive care unit
IGF	insulin-like growth factor
IRMA	immunoradiometric assay
IV	intravenous, intravenously
Jak	janus kinase
KCS	keratoconjunctivitis sicca
kbp	kilo base pair
kDa	kilodalton
l.	latin
LCR	locus control region
LH	luteinizing hormone
LHβ	luteinizing hormone beta subunit
LHX4	LIM class homeodomain transcription factor 4
LIF	leukemia inhibitory factor
LIFR	LIF receptor
LPH	lipotropin
MAPK	mitogen-activated protein kinase
ME	median eminence
MEN	multiple endocrine neoplasia
MRI	magnetic resonance imaging
mRNA	messenger RNA
NeuroD1	neurogenic differentiation factor D1, Beta2
NIL	neurointermediate lobe
NL	neural lobe
o,p'-DDD	2,4'-dichlorodiphenyldichloroethane, mitotane
PAS	periodic acid-Schiff stain (reaction)
P/B	Pituitary height / brain area
PCR	Polymerase chain reaction
PD	*pars distalis adenohypophysis*
PDH	pituitary-dependent hyperadrenocorticism
PI	*pars intermedia adenohypophysis*
Pit-1	POU domain, class 1, transcription factor 1
PO	*l. per os*; by mouth; orally
POMC	pro-opiomelanocortin
PRL	prolactin
PROP-1	prophet of PIT1
PT	*pars tuberalis adenohypophysis*
PTTG	pituitary tumor transforming factor

Rb	retinoblastoma
RER	rough endoplasmatic reticulum
RIA	radioimmunoassay
RNA	ribonucleic acid
RPS19	ribosomal protein S19
RT	reverse transcriptase
RXR	retinoic X receptor
SCN	suprachiasmatic nuclei
SF-1	splicing factor 1
SHH	sonic hedgehog
SNP	single nucleotide polymorphism
Smad1	Small mothers against decapentaplegic homolog 1
SRC-2	steroid receptor coactivator 2
SS	somatostatin
STAT	signal transducer and activator of transcription
STT	Shirmer tear test
Tbx19	T-box transcription factor 19, Tpit
TEF	thyrotrope embryonic factor
Tpit	pituitary T-box transcription factor, Tbx19
TRH	thyrotropin-releasing hormone
TSH	thyroid stimulating hormone
TSH-β	thyroid stimulating hormone beta
UCCR	urinary corticoid-to-creatinine ratio
WNT	wingless/integrated protein

CHAPTER 1

Scope and aims of the thesis

Scope of the thesis

Pituitary-dependent hyperadrenocorticism (PDH), in humans called Cushing's disease, is one of the most common endocrinopathies in the dog, which is caused by an adrenocorticotropic hormone (ACTH)-secreting (corticotroph) pituitary adenoma. **Chapter 2** reviews pituitary morphology, histology and ontogenesis. Furthermore, there is a review on canine PDH and pathogenetic studies performed on ACTH-secreting adenomas in humans and dogs. This thesis contains two main parts. Part I covers follow-up studies and prognostic factors on long-term outcome in dogs that have undergone transsphenoidal hypophysectomy for the treatment of PDH. Part II includes molecular biological studies on the corticotroph adenomas and a study on pituitary dwarfism in German Shepherd dogs.

Part I

Pituitary surgery is the treatment of choice in humans with Cushing's disease. Parallel to this, with the guidance of computed tomography of the pituitary gland, the technique for transsphenoidal hypophysectomy has successfully been applied and refined for the treatment of PDH in dogs at the Department of Clinical Sciences of Companion Animals, Utrecht University. Results from initial 3.5 and 5-year follow-up studies after surgery were promising. In **Chapter 3** the long-term (10-year) surgical outcome in 150 dogs was evaluated and the influence of pituitary size was analyzed with respect to survival and disease-free periods and development of postoperative complications.

Although transsphenoidal hypophysectomy is effective for the treatment of PDH, there are recurrences. Therefore, prognostic factors were searched for among preoperatively available parameters of 181 dogs with PDH (**Chapter 4**). Additionally, in parallel to what is done in humans after pituitary surgery, the postoperative urinary cortisol excretion was evaluated in relation to the period in which the dogs remained in remission.

Measurement of plasma concentrations of pituitary hormones after the administration of releasing hormones at 2 and 8 weeks after surgery, failed to distinguish dogs with recurrences from dogs that remained in remission. Therefore, in **Chapter 5**, the 6-hour plasma profiles of ACTH, α-melanocyte-stimulating hormone (α–MSH), cortisol and growth hormone (GH) before and after surgery were analyzed to see if there is a difference in secretory behavior. In **Chapter 6**, it was investigated whether the immediate peri-operative hormone profiles of ACTH, α-MSH, cortisol and GH were predictive of long-term follow-up results.

Part II

Despite that PDH is a common disease in dogs there are few studies on the molecular biological background. One reason for this may be the well-protected location of the pituitary gland in the skull. Performing a hypophysectomy is, therefore, not only beneficial for the dog but also provides tumor material that can be used for analysis. Expression and mutation analyses were performed for three factors that are important for normal corticotroph differentiation during pituitary organogenesis; pituitary T-box transcription factor (Tbx19/Tpit) (**Chapter 7**), neurogenic differentiation 1 (NeuroD1) (**Chapter 8**), and leukemia inhibitory factor (LIF) and LIF receptor (LIFR) (**Chapter 9**). Leukemia inhibitory factor is an important factor for corticotroph cell differentiation and inhibits the differentiation of the other pituitary cell lineages. Therefore, LIFR was also investigated as candidate factor for pituitary dwarfism in German Shepherd dogs, which is characterized by a

combined anterior pituitary deficiency with hypofunction of the pituitary hormone secreting cells and normal function of the corticotrophs (**Chapter 10**). The findings of the studies are summarized and discussed in **Chapter 11**.

Aims of the thesis

► To analyze the long-term results of transsphenoidal hypophysectomy and investigate whether there are predictors for surgical outcome.
► To perform molecular biological studies on the pituitary adenomas with regard to factors that promote the normal differentiation of corticotroph cells during pituitary organogenesis.

CHAPTER 2

General introduction

Pituitary-dependent hyperadrenocorticism (PDH) in the dog is caused by adrenocorticotropic hormone (ACTH) secreting (corticotroph) pituitary adenoma. This introduction will provide an overview of pituitary gland morphology, function and ontogeny, PDH in dogs and pituitary tumorigenesis.

2.1 Morphology of the pituitary gland

The pituitary gland (*hypophysis cerebri*) is a small appendicular structure, situated on the ventral midline aspect of the diencephalon, suspended from the hypothalamus by a cylindrical stalk (Figure 1). There is no real sella turcica in the dog, the pituitary is positioned in a shallow depression in the basisphenoid bone (*fossa hypophysalis*) (Figure 1B). The pituitary is situated outside the blood-brain barrier, enveloped in dura mater, which also encloses the venous cavernous and intercavernous sinuses that are lying lateral and caudal to the pituitary gland (Figure 2).[170]

In comparison to the human pituitary gland, the long axis of the canine pituitary is tilted to a position almost parallel to the ventral surface of the brain, which gives the impression of being dorsoventrally compressed and the shape of a tear-drop (Figure 1C). Its rostro-caudal length of 10 mm (range, 7-11 mm) is greater than its depth (height) of 5 mm (range 5-7 mm) and width of 7 mm (range, 6-10 mm). The width of the widest part almost equals that of the length (Figure 1D). With increasing body size an absolute increase in pituitary gland size is found, but a relative decrease in proportion to body weight.[109]

The pituitary gland consists of two embryologically and functionally distinct structures, the ectodermal adenohypophysis (*Lobus anterior*) and the neuroectodermal neurohypophysis (*Lobus posterior*). The adenohypophysis, which represents the majority of the entire pituitary gland, can further be divided into three functional parts the *pars distalis*, *pars intermedia* and *pars infundibularis* (pars tuberalis) *adenohypophysis* (Figure 1B). The *pars distalis adenohypophysis*, accounts for the major part of the pituitary gland, lying mainly ventrorostral to the neurohypophysis, and unlike the situation in humans, surrounds the *pars distalis neurohypophysis* to a variable extent, resulting in an "egg-in-the-nest" appearance. The *pars intermedia adenohypophysis* is separated from the *pars distalis adenohypophysis* by the residual lumen of Rathke's pouch, the hypophyseal cleft. The *pars intermedia adenohypophysis* forms a thin layer that coats most parts of *pars distalis neurohypophysis*, from which it is separated only with a delicate layer of vascularized connective tissue. The *pars infundibularis adenohypophysis* (corresponding to pars tuberalis in humans) extends as a collar around the 'pituitary stalk' *(pars proximalis neurohypophysis*) to the median eminence (ME). Into the *pars proximalis neuorhypophysis* (infundibulum) there is an extension of the third ventricle forming a central cavity, the so called infundibular recess or *recessus neurohypophysis.*[109]

The pituitary arterial blood supply comes through the rostral and caudal hypophyseal arteries (Figure 3) that are branches of the arterial cerebral circle (circulus arteriosus cerebri) which mainly is supplied by the internal carotid artery and basilar artery.[109] The blood supply to *pars distalis adenohypophysis* is through the hypophyseal portal system that consists of vessels from a primary blood capillary network of the ME, a secondary blood capillary plexus in the pars distalis and connecting portal venules in the pars tuberalis.[62] The direction of the intra-pituitary circulation and venous drainage is not fully elucidated. The main direction is from the ME down to the pars distalis but there is evidence of blood flow in the other direction.[24] The pars intemedia is relatively avascular but there is a vascular layer at the

junction between the intermedia and neurohypophysis.[106] The *pars neurosa* is richly vascularized. Unlike the situation in the adenohypophysis, the caudal hypophyseal artery enters the caudal *pars distalis neurohypophysis* and branches within the gland into arteries with smooth muscle lining.[109]

2.1.1 Ectopic pituitary tissue

Ectopic pituitary tissue is commonly found at autopsy in humans. Its existence can be explained by the residual pituitary cells left from the stalk of Rathke's pouch that regresses during development of the pituitary gland.[205] A common finding in humans is a small (1-7 mm) piece of ectopic pituitary tissue in the mucosa of nasopharynx, a so called pharyngeal pituitary gland.[47,48] Intracranially located ectopic pituitary tissue sporadically occurs in the dog.[276]

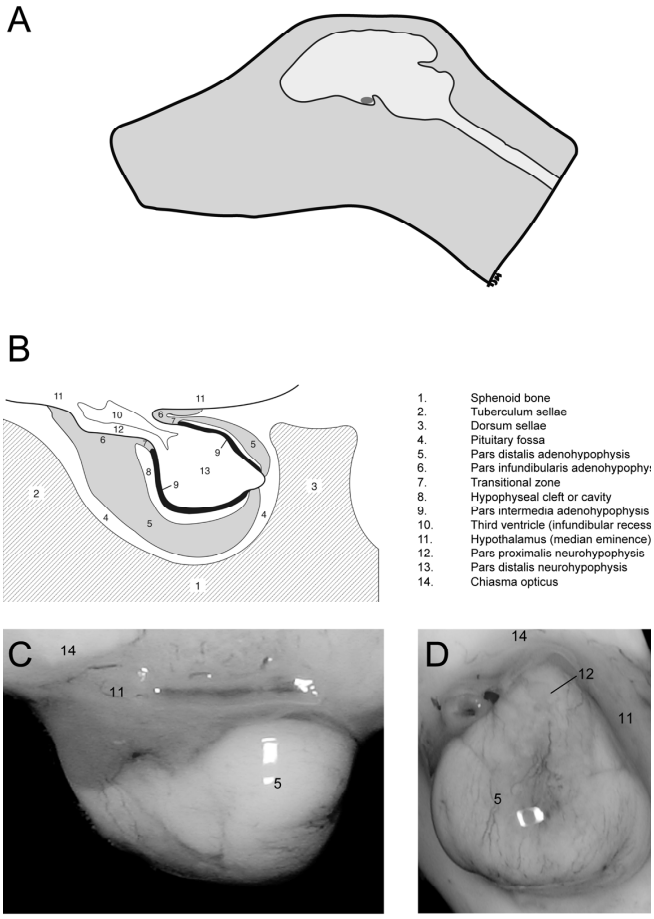
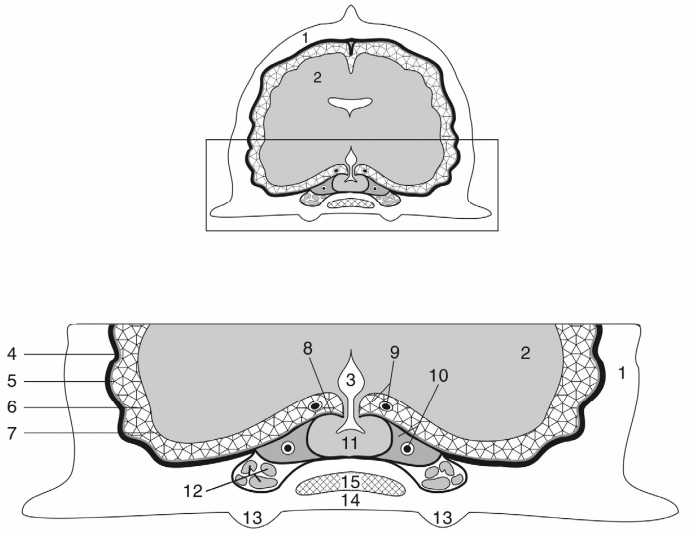
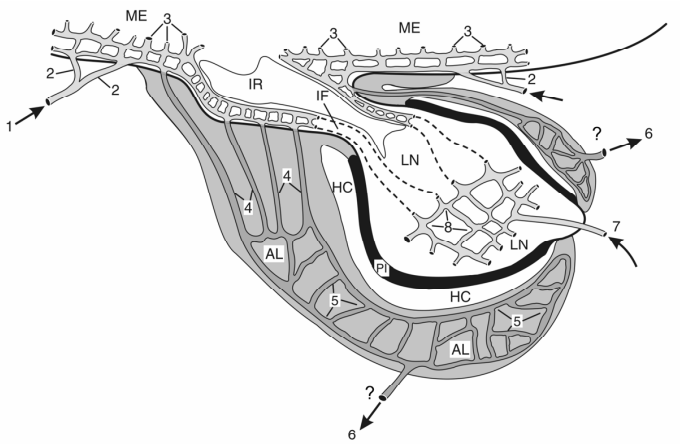


Figure 1. Anatomy of the canine pituitary gland. (A) Schematic lateral view of a canine skull. The pituitary gland (red) is positioned in the cranial cavity underneath the brain. (B) The pituitary gland is lying in the pituitary fossa, which is a shallow hollow in the sphenoid bone. The schematic drawing show the two parts of the pituitary gland; the *adenohypophysis* (grey and black) and the *neurohypophysis* (white) and their respective sub-regions (With permission, Meij 1997) (C) Lateral view of a canine pituitary gland. The pituitary gland is positioned outside the blood-brain barrier but in contact with the hypothalamus, and in cloce vicinity of the optic nerves. (D) Ventral view of a canine pituitary gland, showing the tear-drop form of the pituitary gland. The majority of the visible tissue originates from *pars distalis adenohypophysis*. A small part of the *pars proximalis neuronhypophysis* is seen as a whitish circle at the apex (see color section).



1 = Cranium
2 = Cerebrum
3 = Third ventricle
4 = Dura mater
5 = Arachnoid membrane
6 = Subarachnoid space and trabeculae
7 = Pia mater
8 = Diaphragma sellae
9 = Basal cisterna with caudal communicating a.
10 = Cavernous sinus with internal carotid a.
11 = Hypophysis
12 = Nerve groove for ophthalmic, abducent, trochlear, and oculomotor nerves
13 = Pterygoid bone
14 = Sphenoid bone
15 = Sphenoid sinus

Figure 2. Schematic transverse view of canine skull thorugh the center of the pituitary fossa and a close-up view of the hypophysis in relation to the meninges (with permission, Meij 1997).



1 = Rostral hypophyseal a.
2 = Mantle plexus
3 = Primary blood capillary network
4 = Hypophyseal portal vessels
5 = Secondary blood capillary network
6 = To cavernous sinus
7 = Caudal hypophyseal a.
8 = Neurohypophyseal capillary bed
ME = Median eminence
IF = Infundibulum
IR = Infundibular recess
AL = Anterior lobe
HC = Hypophyseal cleft
PI = Pars intermedia
LN = Lobus nervosus

Figure 3. Schematic lateral view of the vascularization pattern of the hypophyis. The venous drainage from the *pars distalis adenohypophysis* is not fully elucidated. The position of the venous outflow (6) may therefore be another (with permission, Meij 1997).

2.1.2 Histological and ultrastructural appearance of the pituitary gland

Albeit the small size and miniscule appearance, the pituitary gland has a central functional role in maintaining a steady physiologic state, the so called homeostasis. The adenohypophysis is mainly composed of hormone-secreting endocrine cells and the neurohypophysis of hormone-containing axon terminals belonging to hypothalamic nuclei.

2.1.2.1 Adenohypophysis

2.1.2.1.1 Pars distalis adenohypophysis

There are five distinct types of endocrine cells of the pars distalis adenohypophysis classified according to the tropic hormones they produce: the corticotroph secretes adrenocorticotropic hormone (ACTH) and β-lipoprotein (β-LPH), the thyrotroph thyroid stimulating hormone (TSH), the gonadotroph luteinizing hormone (LH) and follicle-stimulating hormone (FSH), the somatotroph growth hormone (GH), and the lactotroph prolactin (PRL) (Table 1). Based on histological staining characteristics the cells are divided into basophilic cells (corticotrophs, thyrotrophs and gonadotrophs), and acidophilic (somatotrophs and lactotrophs) (Figure 4 A, B). In addition to the endocrine cells, there are different types of chromophobic cells, so called folliculo-stellate cells that do not immunostain for the pituitary hormones. The folliculostellate cells can further be divided into subgroups; the stellate cells with fine processes interspersed between the other cells of the *pars distalis*, the follicular cells of unknown origin and significance, lining follicles, and a third cell type with only a few specific granules considered to be a resting degranulated form of the other granulated cells [62]. Also, there are pituitary stem-cells present in the pars distalis that remain to be identified histopathologically.[4]

Table 1
The hormone-producing cells of the adenohypophysis

Endocrine cell	Hormone(s)	% cells in pd
Corticotroph	ACTH, β-LPH	10
Thyrotroph	TSH	10
Gonadotroph	LH, FSH	15
Somatotroph	GH	50
Lactotroph	PRL	15
Melanotroph	α-MSH, CLIP	

ACTH = adrenocorticotropic hormone; α-MSH = α-melanocyte-stimulating hormone; β-LPH = β-lipoprotein; CLIP = corticotropin-like intermediate lobe peptide; FSH = follicle-stimulating hormone; GH = growth hormone; LH = luteinizing hormone; PD = pars distalis adenohypophysis; PRL; prolactin; TSH = thyroid stimulating hormone

As in other endocrine organs, connective tissue is scarce and capillaries abundant.[109] In contrast to what sometimes is believed, there are also nerve fibers in the *pars distalis adenohypophysis*. They are thought to be sympathetic in nature and to occur around vessels and therefore have little to do with the endocrine activities. Also, peptidergic fibers have been identified in the *pars distalis adenohypophysis* of the dog, rat and monkey. The nerve fibres are closely related to the glandular cells with synaptic contacts demonstrated between them. The origin and functional significance of theses peptidergic fibers are not known.[96,114]

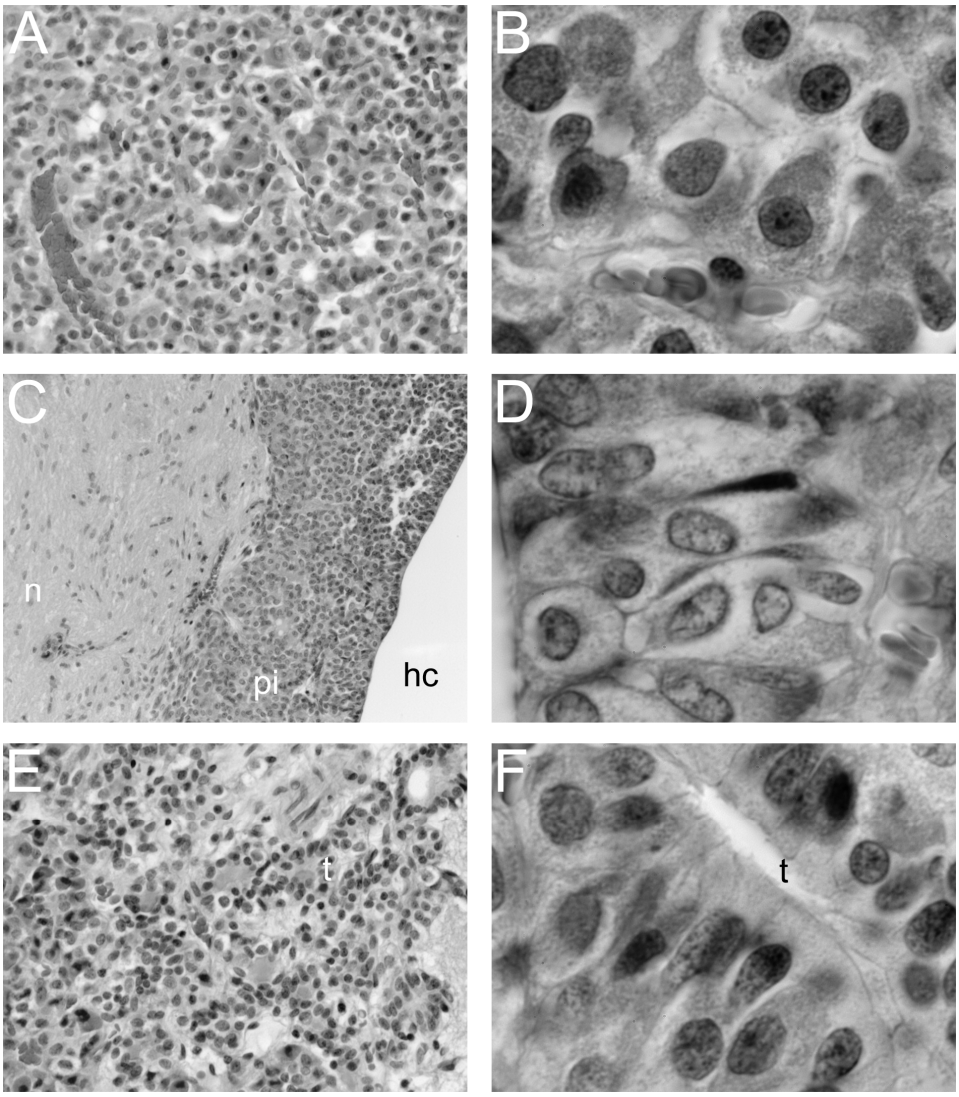


Figure 4. Histological pictures of the canine *adenohypophyis*, stained with hematoxyline and eosin. (A, B) The *pars distalis adenohypophysis*. The chromophilic (acidophils (red) and basophils (blue)) and chromophobic cells are shown and the rich amount of vascular sinusoids in the tissue. (C) *Pars intermedia adenohypophysis* (right), outlining the neurohypophysis (left) and facing the hypophyseal cleft (emty space at the right). (D) the cells of the *pars intermedia adenohypophysis* in high magnification. (E, F) *Pars infundibularis adenohypophysis* (pars tuberalis). The cells are forming small tubular stuctures in this distinct and richly vascularized part of the adenohypophysis. (Magnification 200x (C), 400x (A,E), 1000x (B, D, F) (see color section).

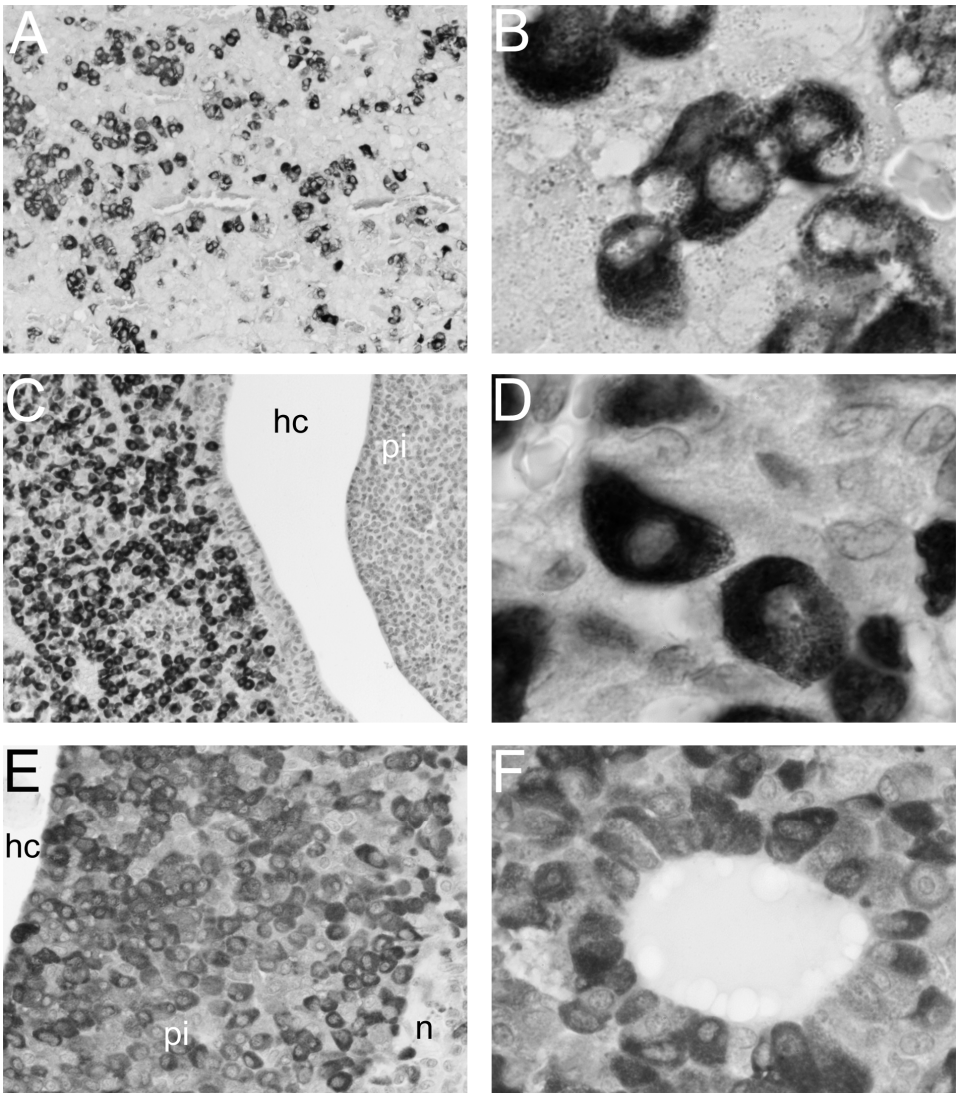


Figure 5. Immunohistological stainings of pituitary hormones. (A, B) Staining for adrenocorticotropic hormone (ACTH) showing the corticotrophs of the *pars distalis adenohypophyis*. (A) The corticotrophs are dispersed through the pituitary tissue, mainly lying in small groups. A higher concentration of corticotrophs and larger groups are seen in the distal parts of the *pars distalis adenohypophysis* (left). (B) The corticotroph cells at high magnification (1000x). (C, D) Staining for growth hormone (GH) showing the somatotrophs that are the predominant cells at the dorsal aspect of the *pars distalis adenohypophysis* facing the hypophyseal cleft and pars intermedia (right). (D) Somatotroph cells at high magnification (1000x). (E, F) Staining for α-melanocyte stimulating hormone (α-MSH) on *pars intermedia adenohypophysis*. (F) A typical colloid filled follicle of the *pars intermedia adenohypophysis* (see color section).

In the dog, the somatotrophs account for 50% or more of the endocrine cells in *pars distalis adenohypophysis*, the other cell types about 5-15% each[216] (Table 1). The proportion of cells changes on basis of age, gender, physiological and pathological status, which results in great variation in the relative proportions between individuals.[152] For example, during lactation the proportion of acidophilic cells increases and remains elevated until the end of lactation constituting approximately 65 % of the cells. After lactation their numbers return to pre-pregnancy levels.[109] In cases of loss of negative feed-back inhibition from the hormones released by the target organ, the pituitary cells undergo a considerably hypertrophy. [62]

The endocrine cells are oriented along the sinusoids. In general, the basophilic cells are located at the ventral, peripheral parts of the *pars distalis adenohypophysis*, and the majority of acidophilic cells (the somatotrophs) at the dorsal parts of the *pars distalis adenohypophysis*. The basophilic cells are larger than the acidophilic cells, measuring approximately 20 μm in diameter and have a more elongated shape.[109]

The endocrine cells of the *pars distalis* differ in their ultrastructural characteristics. However, there are only a few electron microscopy reports on the ultrastructure of the canine pituitary gland.[88] The ultrastructural descriptions below, are based on studies performed in other species.[52] The corticotrophs are spread throughout the pituitary and are generally arranged in small groups (Figure 5A, B). They occur in higher concentrations and larger groups, in the ventrocentral and cranial portions of the pituitary gland[69,170] (Figure 5B). Ultrastructurally, the corticotrophs are irregular shaped with an eccentric nucleus bearing indentations. The granules, 150-200 nm in diameter, are less abundant than in other cell types and appear near the cell surface and in association with the extensive Golgi apparatus, in which immature granules of low density may be seen. An extensive rough surfaced endoplasmatic reticulum (RER) and few marginal granules are indicative of rapid turnover. There are small stacks of cisternae and small vesicles dispersed throughout the cytoplasm, which also contains free ribosomes, spherical to elongated mitocondria, and sometimes a few lysosomes[52] (Figure 6). Adrenalectomy results in dilatation of the RER cisternae with the containing low-density material creating a spongy cell. Cortisol treatment causes involution of the RER, the Golgi apparatus and the cell size whereas the number of lysosomes in the corticotrophs increases.[52] The melanotrophs are mainly located in the *pars intermedia* (see below), but can occasionally occur as single cells in the *pars distalis adenohypophysis*.

The thyrotrophs appear single or arranged in small groups, mainly in the ventrocentral, paramedian plane of the *pars distalis adenohypophysis.*[73,170] Ultrastrucutrally, the thyrotropes are irregular shaped or angular cells similar to the corticotrophs. The granules of the thyrotropes are somewhat smaller (100-150 nm) than those of the corticotrophs.[52]

The gonadotrophs are the largest endocrine cells of the pituitary gland. Gonadotrophs are distributed throughout the pars distalis but with the highest concentration in the dorsocranial part where they may appear in clusters.[70,170] Following castration, the gonadotrophs enlarge considerably, and form so called ‘castration cells’. Eventually, ‘signet ring” cells are formed with a peripheral rim of cytoplasm surrounding one or several large vacuoles.[62] Ultra-structurally, the gonadotrophs are characterized by numerous amounts of granules (100-200 nm), an extensive RER with dilated cisterns, and a typical spherical Golgi apparatus.[52]

The somatrotrophs are the most abundant hormone producing cells of the pars distalis, with the highest concentrations in the dorsal region (i.e., in the vicinity of the *pars intermedia*[74,170] (Figure 5 C,D). They appear in equal amounts in both females and males.

Ultrastructurally, the somatotrophs have abundant large sized granules (300-600 nm) and a prominent Golgi apparatus.[52]

The lactotrophs synthesize and secrete prolactin (PRL) and are more abundant in the female than in the male pituitary gland. The lactotrophs occur as single cells or in small groups. The lactotrophs show much a similar distribution pattern as the corticotroph cells, being most abundant ventrocentrally and in the cranial portions of *pars distalis.*[71,170] Ultrastructurally, the lactotrophs have granules in the lowest number, but the largest size (600-900 nm). The cells are larger in females than in males.[52]

The transitional zone of the canine pituitary is well developed and contains corticotrophs similar to those in *pars distalis adenohypophysis*.

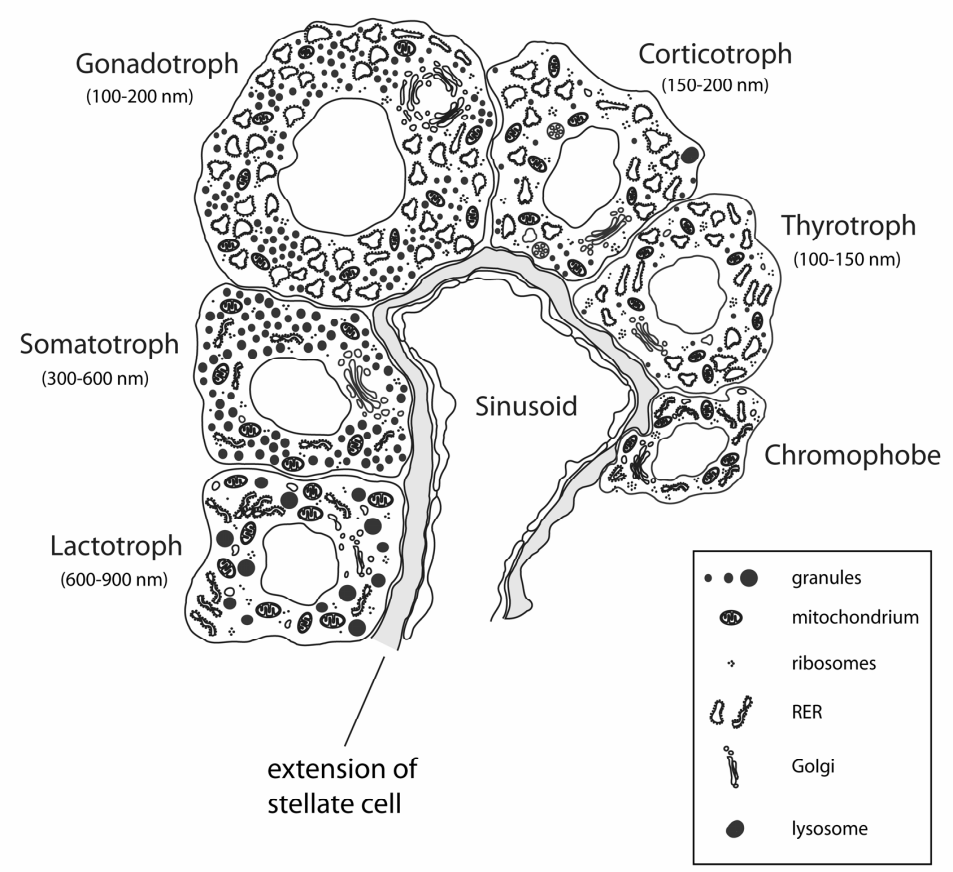


Figure 6. Schematic drawing of the ultrastructure of the endocrine cells of the pars distalis adeno-hypyphysis. The endocrine cells are surrounding the sinusoids from which they are separated from through a thin arm of the stellate cell (grey) (Modified from Constantinides 1974, Functional electronic histology - A correlation of ultrastructure and function in all mammalian tissues. Amsterdam, Elsevier Scientific Publishing Company).

2.1.2.1.2 Pars intermedia adenohypophysis

The thin layer of the *pars intermedia adenohypophysis* almost completely envelops the *pars distalis neurohypophysis*. The *pars intermedia* blends with the *pars distalis adenohypophysis* at the distal reflection. At the proximal transition zone, the folded epithelium projects into the lumen of the hypophyseal cleft. These folds are more prominent in the brachycephalic breeds.[109] The cells of the pars intermedia are commonly arranged as a pseudostratified epithelium, with approximately 15-20 layers of cells. However, the thickness varies considerably. The basement membrane between the pars intermedia and the neurohypophysis is often defective in parts, which short villus-like penetrations of intermedia cells into the neural tissue.[109] Follicles may occur, (Figure 5F) containing amorphous periodic acid-Schiff (PAS) positive material. The surface of the pars intermedia facing the hypophyseal cleft may be covered with a single layer of low cuboidal cells.[106] Nerve fibres, described already in 1894,[106] penetrate the *pars intermedia* (Figure 5E).

The canine *pars intermedia adenohypophysis* (Figure 4C, D, 5E, F) contains two endocrine cells; the melanotrophs (A cells) and corticotrophs (B cells). Both cells mainly appear as chromophobic cells. The melanotrophs are the most predominant, comprising more than 90% of the cell population that stain strongly for α-melanocyte-stimulating hormone (α-MSH) (Figure 5E) and weakly for ACTH. The B cells resemble the corticotrophs of the *pars distalis adenohypophysis* and stain intensely for ACTH but not for α-MSH.[97] Ultrastructurally, the melanotrophs are polyhedral cells with nearly spherical nucleus containing cytoplasmic granules of varying size (300-1000 nm in diameter) and electron density. The B cells are less frequent and contain smaller granules that very much resemble the cells of the *pars distalis* (Figure 7C). A third agranular interstitial cell is described, which surrounds a highly electron-dense colloidal material has microvilli on the apical surface that is in contact with the colloid.[138,201]

2.1.2.1.3 Pars infundibularis adenohypophysis (Pars tuberalis)

The *pars tuberalis* consists of clusters of epithelial cells that are often lying arranged in small follicles (Figure 4E, F), transversed by wide hypophysial portal venules. The most numerous cells are specific secretory cells, which express numerous melatonin receptors and are thought to play a role in the seasonal reproductive cycle and also pulsatile release of the pituitary hormones (see below).[62] Ultrastructurally, the secretory cells are different from the agranular follicular cells. Occasionally, cells of the *pars distalis adenohypophysis* may occur in the *pars tuberalis* as small clusters of corticotrophs, melanotrophs, gonadotropes and thyrotropes.[63,69,170]

2.1.2.2 Neurohypophysis

From the hypothalamic supraoptical and paraventricular nuclei originate unmyelinated axons, forming the hypothalmohypophyseal tract (Figure 8) that runs through the stalk (infundibulum) of the neurohypophysis to the *pars distalis neurohypophysis*. In the axon ends secretory granules accumulate consisting of antidiuretic hormone (ADH) (arginine-vasopressin, AVP) and oxytocin. Upon stimulation, the granules are secreted by exocytosis into the blood vessels of the posterior lobe. The andidiuretic hormone is produced in the cell bodies as a prohormone, which is processed during transport through the axons and stored in neurosecretory granules of 100-180 nm in diameter, the so called Herring bodies, consisting of ADH-neurophysin (carrier protein) complex within a phospholipid membrane.[109]

The axons are supported in an extensive neuropil by the pituicytes, which are mainly glial cells, also called gliocyti centrales.[109] In addition, there are some microglial cells present.[62]and evidence for sympathetic innervation of the *pars distalis neurohypophysis* .[109]

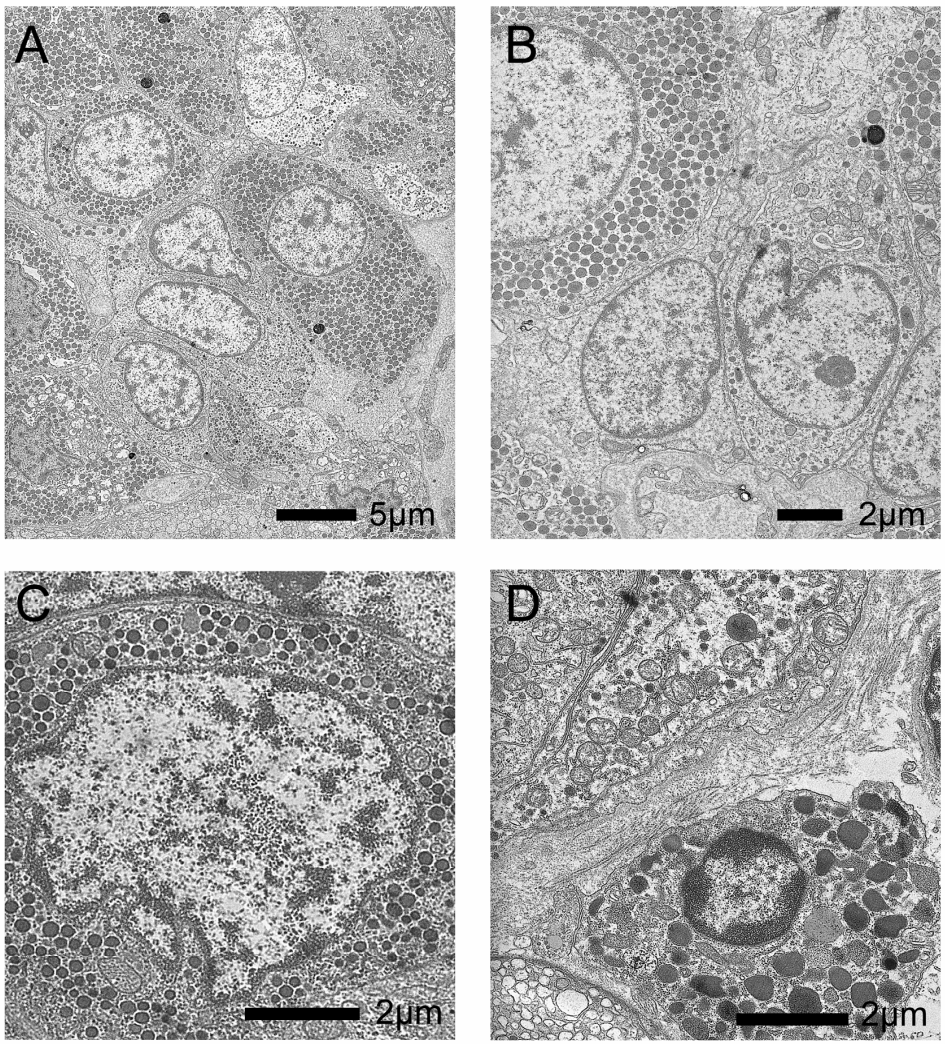


Figure 7. Electron microscopy pictures from a canine pituitary. (A) Overview of the arrangement of the cells of pars distalis adenohypophysis. (B) Close-up view of a polyhedral cell with a low number of peripheral small-sized secretory granules, that may a thyrotroph of corticotroph. (C) A corticotroph-like cell of the pars intermedia. (D) Another cell of the pars intermedia with large granules that may be a melanotroph.

2.2 Function of the pituitary gland

The hypothalamic-pituitary axis has a central role in the neuroendocrine system in the control of vital functions such as stress response, basal metabolism, growth, reproduction, and lactation.[215] The hypothalamic-pituitary axis consists of three major systems, a neuronendocrine system connected to an endocrine system by portal circulation (*pars distalis adenohypophysis*), a neurosecretory pathway (*neurohypophysis*), and a direct neural regulation of endocrine secretion (*pars intermedia adenohypophysis*) (Figure 8A).[215]

The hormone secretion from the *pars distalis adenohypophysis* is regulated by an intimate contact between the hypothalamus and pituitary gland maintained via the hypophysial portal system. At the ME, hypothalamic neurons secrete their respective regulatory peptides corticotrophin-releasing hormone (CRH), thyrotrophin-releasing hormone (TRH), gonadotrophin-relasing hormone (GnRH), GH-releasing hormone (GHRH) and somatostatin (Figure 8B) from axon terminals into the primary capillaries of the portal system and subsequently transported by the hypothalamo-pituitary portal vasculature to the sinusoids of *pars distalis adenohypophysis* and their respective target cells. Hormone release from the canine *pars intermedia adenohypophysis* is under strong hypothalamic tonic dopaminergic inhibition[194] that can be reversed by the administration of a dopamine antagonist such as haloperidol.[123]

The pituitary hormone release is subject to negative feed-back (closed-loop) system. In a long-loop feed-back system the hormone of the target endocrine organ acts on the pituitary and the hypothalamus. In a short-loop system, the pituitary hormone regulates its own secretion directly by acting on the hypothalamus.[216] Additionally, there is evidence of a ultra-short loop the hormone acts within the pituitary gland through paracrine or autocrine communications[32,210] (Figure 8B). Superimposed on this blood-borne regulatory mechanism there are other signals mediated by neurotransmittors and hypophysiotropic hormones that represents the influence of environment, stress and intrinsic rhythmicity.[216]

2.2.1 The pituitary-adrenal-axis and the regulation of synthesis and secretion of pro-opiomelanocortin (POMC) peptides

The main topic of the present thesis is pituitary-dependent hyperadrenocorticism (PDH), which is caused by a pituitary tumor secreting ACTH, and sometimes α-melanocyte stimulating hormone (α–MSH). Therefore, a detailed description of the regulation of these cells will be given.

Both ACTH and α-MSH are products of post-translational cleavage of a common precursor molecule, the pro-opiomelanocortin (POMC). The differential processing of POMC in the two cells is dependent on the expression of prohormone convertase 1 (PC1) and PC2. The expression of PC1 generates ACTH in the corticotrophs and co-expression of PC1 and PC2 generates α–MSH in the melanotrophs.[23,296] Also, the post-translational cleavage gives rise to a number of other peptides that are co-released with ACTH and α-MSH (Figure 9 A).

The major hypothalamic stimulating factors for ACTH release is corticotropin-releasing factor (CRH) and AVP.[75,121] Hormone release from the *pars intermedia* is mainly neurongenically regulated by the influence of a tonic dopaminergic inhibition exerted by axon terminals of hypothalamic nuclei. Furthermore, inhibition is exerted through GABA positive neurons have an inhibitory effect and by delta-opoid receptor agonists. Release of α-MSH

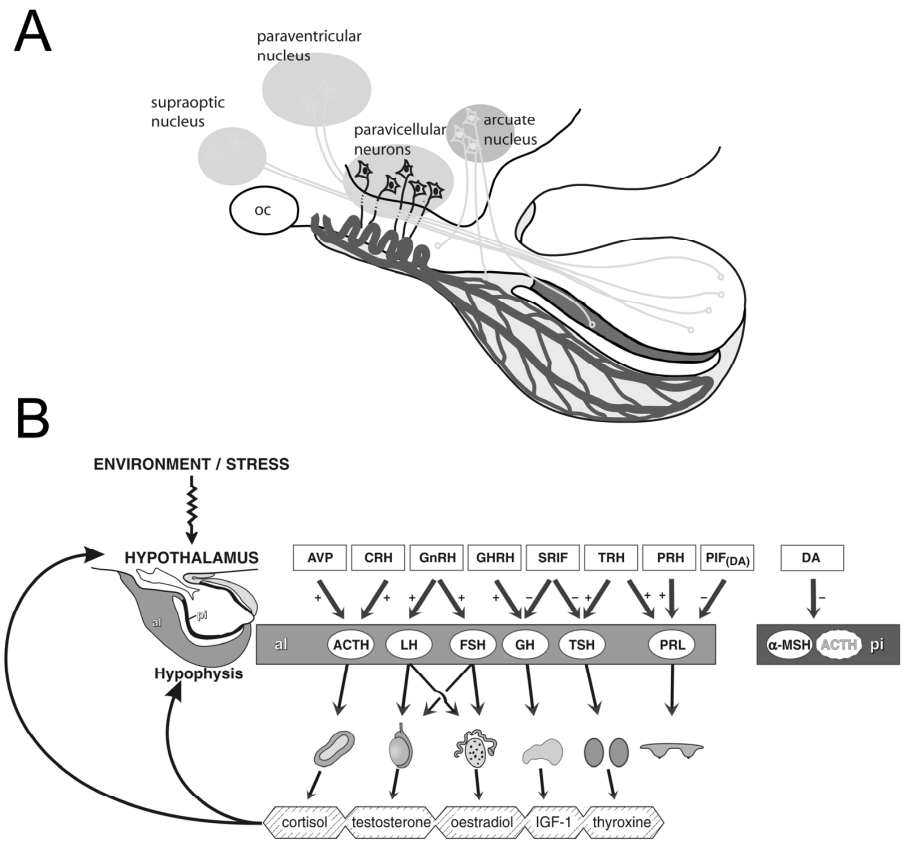


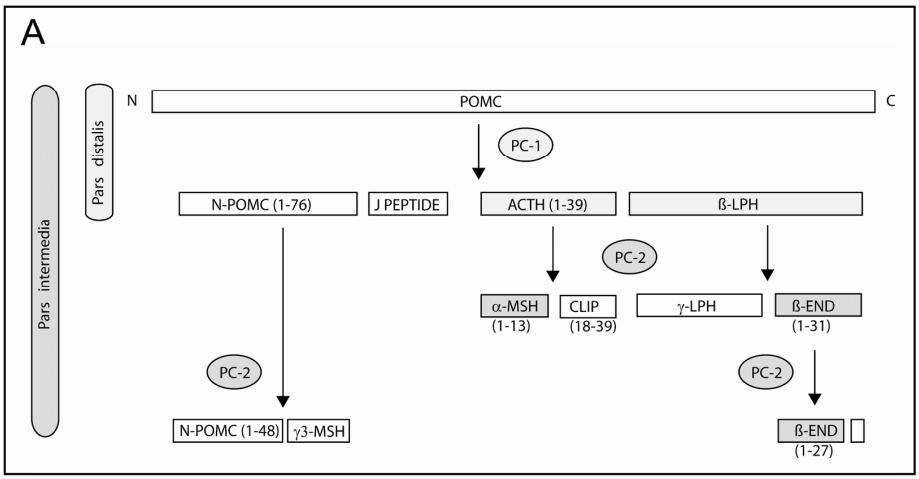
Figure 8. Hypothalamo-pituitary axis (A). Hypophysiotrophic nuclei in the parvicellular area (gray area) releases their regulatory hormones into the primary capillary plexus of the ME from where they are transported via portal veins in pars tuberalis to the secondary blood capillary plexus (red) of pars distalis adenohypophysis. The hypothalmaic nuclei (yellow) in the supraoptic and paraventricular areas are sending unmyelinated axons constituting the hypothalamo-pituitary tract, to the neurohypophysis where they store and secrete oxytocin and vasopressin. Hormone release from the neurohypophysis is mainly through release of oxytocin (Modified, Dellman 1998[62]).

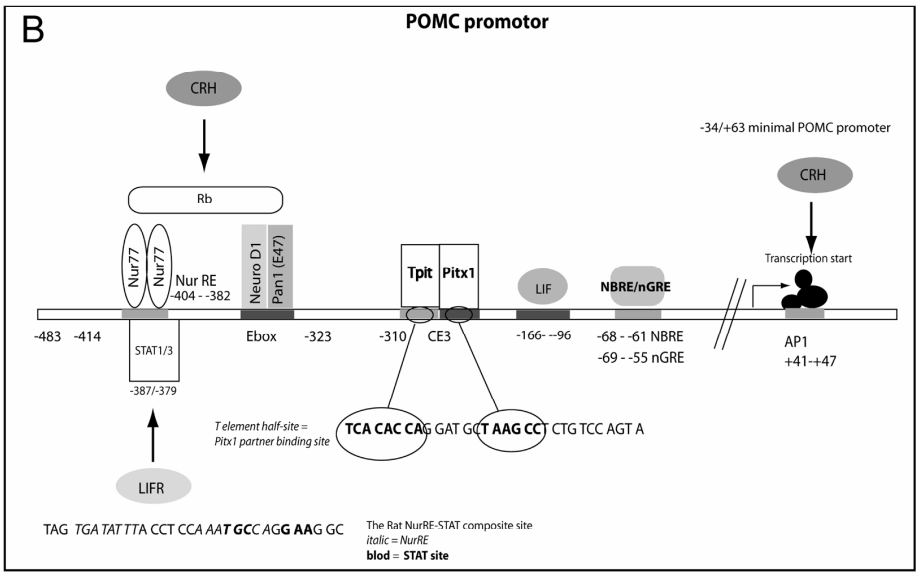
Simplified diagram of hypophysiotropic regulation of the secretion of hormones in the adeno-hypophysis (B). pd: *pars distalis adenohypophysis*; pi: *pars intermedia adenohypophysis*; AVP: arginine vasopressine; CRH corticotropin releasing hormone; GnRH: Gonadotropin releasing hormone; GHRH: growth hormone releasing hormone; SRIF: somatostatin; TRH: thyrotrophin releasing factor; 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 growth-factor 1 (Modified, Kooistra 2000)

can be induced with beta-adrenergic stimulation, serotonin, CRH (in vitro), met-enkephalin (*in vitro*) and my-opoid receptor agonists, as reviewed by Saland and co-workers.[225] Despite a high concentration of bioactive ACTH in the canine *pars intermedia*,[97] it is discussed whether the corticotrophs of the pars intermedia contributes to the plasma concentrations of ACTH. ACTH secretion from perifused cells from the neurointermediate lobe in vitro has been detected,[125] and there is evidence that the *pars intermedia adenohypophysis* contributes to some, however small part, of the plasma ACTH concentration.[132] The ultrastructural appearance of these cells, in contrast to the situation in the *pars distalis adenohypophysis* is also indicative of less active hormone secretion.

The glucocorticoids exerts is negative inhibition at both the pituitary and hypothalamic level resulting in reduction in transcription of the ACTH precursor, pro-opiomelanocortin. The efficiency of the glucocorticoid feed-back is not static but depends on the stressor stimulus.[157] The POMC-producing cells of the pars intermedia adenohypophysis are resistant to glucocorticoid suppression, due to the absence of glucocorticoid receptors on these cells as a result of tonic hypothalamic inhibiting influence on the expression of glucocorticoid receptors.[8,9]

Figure 9. *Posttranslational cleavage of pro-opiomelanocortin (POMC), by the action of prohormone convertases 1 (PC1) and 2 (PC2)* (A). Enzymatic action of PC1 results in the cleavage products adrenocorticotrophic hormone (ACTH) and β-lipotropin (β-LPH). In the pars intermedia the presence of PC2 in high concentrations result in further cleavage into α-melanocyte-stimulating hormone (α –MSH) and β-endorphin (β-END). C = carboxy-terminal of the protein; CLIP = corticotropin-like intermediate lobe peptide J Peptide = joining peptide; N = amino-terminal of the protein (Modified from Rijnberk 1996[216] and Meij 1997[170] and Zou et al 1992[296]).
Regulation of POMC transcription (B). The corticotrophin releasing hormone (CRH) inducible nuclear orhphan steroid receptor Nur77 (NGF-1, TR3, Nr4a1) and leukaemia-inhibitory factor (LIF) inducible signal transducer and activator of transcription (STAT) 1/3 heterodimer binds at the Nur-repsonsive element (RE)-STAT site.[148,162] The basic helix-loop-helix heterodimer of the corticotroph specific neurogenic differentiation factor D1 (NeuroD1) also called Beta2, and Pan1 (E47) binds at the Ebox region.[207] The retinoblastoma (Rb) interacts with NeuroD1 and form a bridge between NeuroD1 and Nur77 [21]. The pituitary T-box transcription factor (Tpit, Tbx19) interacts with and is obligate for Pitx1 induction of POMC transcription.[141,142] The proximal LIF responsive subregion lacks element to bind STAT proteins.[147] NBRE = proximal Nur77-binding response element binds Nur77 or Nur1 monomers [187] and overlaps with the negative glucocorticoid responsive element (nGRE). The binding of activating protein 1 (AP1) is promoted by CRH.[30]


A
Pars intermedia
Pars distalis
N
POMC
C
PC-1
N-POMC (1-76)
J PEPTIDE
ACTH (1-39)
ß-LPH
PC-2
α-MSH
(1-13)
CLIP
(18-39)
γ-LPH
ß-END
(1-31)
PC-2
PC-2
N-POMC (1-48)
γ3-MSH
ß-END
(1-27)


B
POMC promotor
CRH
Rb
Nur77
Nur77
Nur RE
-404 - -382
Neuro D1
Pan1 (E47)
Tpit
Pitx1
LIF
NBRE/nGRE
-34/+63 minimal POMC promoter
CRH
Transcription start
-483
-414
STAT1/3
-387/-379
Ebox
-323
-310
CE3
-166- --96
-68 - -61 NBRE
-69 - -55 nGRE
AP1
+41-+47
T element half-site =
Pitx1 partner binding site
TCA CAC CAG GAT GCT AAG CCT CTG TCC AGT A
LIFR
TAG TGA TAT TTA CCT CCA AAT GCC AGG AAG GC
The Rat NurRE-STAT composite site
italic = NurRE
blod = STAT site

The expression of the POMC gene in corticotrophs and melanotrophs is regulated by the same promoter element.[183] Factors that bind to the POMC-promoter region have been identified and is still focus for intensive research to elucidate the exact regulatory mechanisms of this central and important peptide. The pituitary T-box transcription factor (Tpit, Tbx19) is obligate for POMC transcription and loss-of-function mutations in this gene in humans and mice result in early-onset pituitary ACTH deficiency, in humans called isolated ACTH deficiency (IAD).[211] Stimulation with the leukemia inhibitory factor (LIF) and its closest relative cytokines oncostatin M (OSM) also stimulate POMC transcription via the Janus protein tyrosine kinase (JAK) - signal transducer and activator of transcription (STAT) pathway. Also LIF stimulate ACTH secretion, and has synergistic effects with CRH.

The Neurogenic differentiation factor 1 (NeuroD1) is a basic helix-loop-helix transcription factor that is specific for the corticotrophs[140,208] and not expressed in the melanotrophs of the *pars intermedia* of mice.[208] NeuroD1 acts in the adult pituitary gland where it interacts with Pitx1 to synergistically upregulate POMC expression.[207,208] The action of glucocorticoids on the corticotroph cells involves binding of intracellular receptor, attraction of helper molecules and binding to the POMC receptor. Interestingly, paradoxically stimulatory effect is seen in vitro on cells exposed to both glucocorticoids and LIF[148] (Figure 9B).

2.2.2. Biologic effects of POMC-peptides

ACTH stimulates glucocorticoid release from the two inner zones of the adrenal cortex, and promotes growth of these.[290] The released glucocorticoids will subsequently exert their metabolic and antiinflammatory effects.[216] However, ACTH as being a POMC peptide, also has intrinsic anti-inflammatory, metabolic and cardiovascular effects. [240,274]

The aminoacid sequence of α-MSH is highly similar between species.[120] Except for stimulation of the melanocytes resulting in skin darkening, the other biologic effects of α-MSH have long been unknown although the presence of specific receptors have been found in many tissues.[252] However, it has been discovered that α-MSH acts as a potent anti-inflammatory agent and regulates energy metabolism and a mediator for the anorectic effects of leptin.[20] This has resulted in an increased interest for this, former neglected peptide.[42,240]

2.2.3. Pulsatile fluctuations in plasma adenohypophyseal hormone concentrations

Endocrine glands communicate with their target tissues via intermittent chemical signaling. The rhythm of this variation in hormone release is at several levels. There are circannual (seasonal) variations, such as reproductive activity in domestic animals, infradian variations (>24 h) e.g., the gravitational influences of the moon, circadian variations (about 24 h) or ultradian variations (<24 h).[184] There is an apparently intrinsic rhythmicity of small amplitudes (2-10 min) of the pituitary cells, on which an ultradian rhythm is superimposed of hypophysiotrophic releasing factors with or without the concurrent withdrawal of inhibiting factors.[184] The suprachiasmatic nuclei (SCN), which are paired nuclei located in the anterior hypothalamus just above the optic chiasm, is the principal "biological clock" or pacemaker for rhythmicity in mammals.[152,283] The SCN gets light-induced electrical impulses from the eye via the retinohypothalmic tract. The electrical impulses are transmitted to the pineal gland (epiphysis) where it is translated into endocrine signals in form of melatonin secretion.[178,179,184,223] However, there are cells in the *pars tuberalis adenohypophysis* that also

express rhythmic clock genes.[112] Most probably these are the agranular secretory cells that also expresses melatonin receptors.[158] In the dog, melanotonin receptors are expressed in the chromophobe and basophilic cells of *pars infundibularis adenohypophysis* as well as on cords of basophilic cells that extends into the *pars distalis adenohypophysis*.[239]

The circadian rhythm influences plasma concentrations of pituitary hormones as is shown in humans[272] but also pituitary mitotic activity in rat is reported to be influenced by the circadian rhythm.[152] Information on the possible circadian secretion of adenohypophyseal hormones is scarce, and has so far not been detected in the release of POMC peptides,[194] cortisol[137] or TSH[35] in healthy dogs although a clear diurnal rhythm in of melatonin in peripheral blood has been found. All pituitary hormones, however, are released in an ultradian pulsatile fashion in the dog.[31,124,132]

In principle, all released hormone that is secreted is released with secretory bursts and almost no hormone is released 'tonically' between the pulses. The hormone level achieved is dependent amplitude and frequency of pulses of hormone release and the metabolic clearance rate of the hormone[184]. The normal pattern of hormone release may be altered with a concurrent disease, as has been shown for many species for example humans with Cushing's disease.[221,261,273] Fluctuations in plasma hormone concentrations can be studied with the Pulsar computer programme independently of information of eliminations rates[181] in contrast to what is needed for deconvolution techniques often used in human medicine[272]. The Pulsar programme identifies adenohypophyseal hormone pulses based on their amplitude and duration with respect to a smoothed baseline (using a weighted linear regression of hormone level versus time within a certain time window), taking into account the standard deviation of the assay at a given hormone concentration. There are limitations of this method; it is sensitive to influences of high intra-assay coefficients, hormone concentrations measured by immunoasays do not represent the bioactivity of the hormone and measurement at time intervals does not give the true underlying pattern of hormone fluctuations. However, the method is a tool for objective comparison of different data sets. [31]

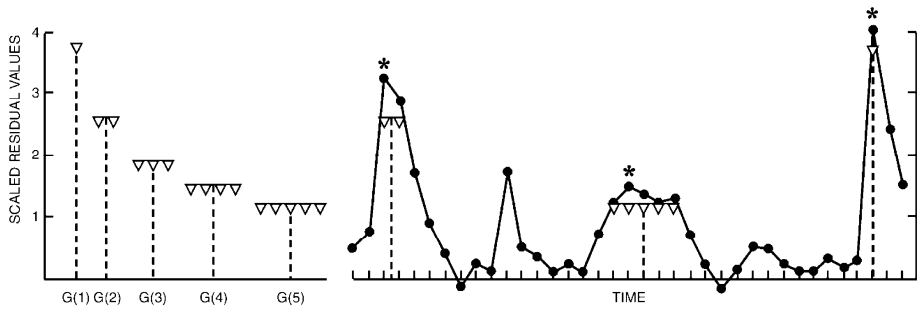


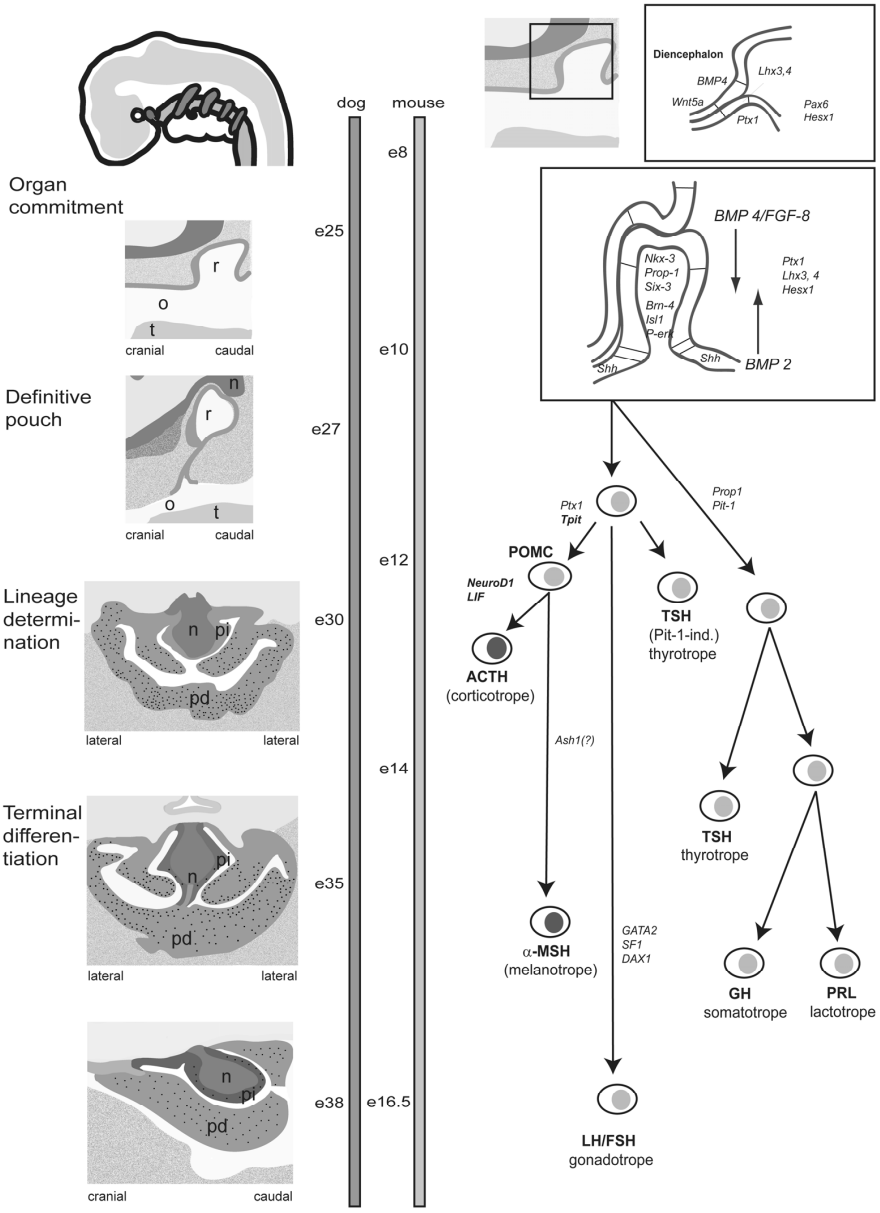
Figure 10. Computer-assisted analysis of pulsatile secretion by comparison of possible peaks against cut-off criteria G(1)-G(5). Before testing, series were stripped of long-term trends and scaled in units of assay standard deviation. Three significant peaks, having 2 successive points greater than G(2), 5 successive points greater than G(5), and 1 point greater than G(1) respectively, meet the cut-off criteria and are marked with asterisks.

2.3 Pituitary ontogeny

The pituitary gland is an evolutionary conserved structure present throughout the vertebrates.[106] During organogenesis, the *adenohypophysis* and the *neurohypophysis* develop from an intimate interplay between the ectoderm of the dorsal aspect of the primitive mouth (stomodeum) and the ventral neuroectoderm of the diencephalon, the primordium of the ventral hypothalamus.[57,58,92,136,227,248,258,281,282] At an early phase, these two ectodermal layers are closely apposed. At the site of apposition, there is a thickening (placode formation) in the stomodeal ectoderm, which then evaginates and forms the adenohyophyseal pouch (Rathke's pouch).[186] The formation of Rathke's pouch is paralleled with a thickening and evagination of the diencephalon (infundibulum). The infundibulum gradually expands and the distal bud develops into the *neurohypophysis*. The point of fusion of the ectodermal layers is maintained, and this part of the Rathke's pouch eventually develops into the pars intermedia. The bulk of adenohypophyseal cells develops through massive proliferation of the anterior wall of Rathke's pouch (Figure 11). In the dog, the Rathke's lumen is divided into two (one dorsal and one ventral lumen) by right and left ledges. In the fully developed pituitary gland, only the dorsal lumen persists as Rathke's cleft.[226] With the growth of the embryo, there is an increasing distance between the ventral brain and dorsal oronasal cavity. Rathke's pouch closes and forms a vesicle. The ectodermal stalk regresses gradually and the separation of the developing *adenohypophysis* and the primitive oral cavity completes by formation of the basisphenoid bone.[41] However, part of the epithelial tissue forming the stalk of Rathke's pouch may persist as a canal (the adenohypophyseal foramen) located in the center of the basisphenoid. The canal is more commonly seen in brachycephalic breeds.[191]

The blood supply to the *adenohypophysis* develops from invading capillaries from the mesenchymal surroundings. This occurs at embryonic day (e) 30 in the dog. The portal vessels develop before the capillary loops in the median eminence (ME), which is illustrated by a rich supply of capillaries in the pars distalis but no detectable capillary loops in ME. There is no evidence of capillary loops until e52 in the dog.[226]

Figure 11. The development of the pituitary gland. Left: pituitary ontogeny of the canine pituitary and the corticotroph cells (black spots in the pars distalis adenohypophysis and darkening of the pars intermedia adenohypophysis) (Adapted from Sasaki and Nishioka[226] and Nussey and Whitehead, 2001[193]). r: Rathke's pouch; o: oral cavity; t: tongue; n: neuron hypophysis; pi: *pars intermedia adenohypophysis*; pd: *pars distalis adenohypophysis*. Center: time bar showing embryonic (e) days of development in the dog and mouse. Right: 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 (see color section).


dog
mouse
e8
e25
e10
e27
e12
e30
e14
e35
e16.5
e38
Organ commitment
Definitive pouch
Lineage determi-nation
Terminal differen-tiation
r
o
t
n
pi
pd
cranial
caudal
lateral
Diencephalon
BMP4
Lhx3,4
Wnt5a
Ptx1
Pax6
Hesx1
BMP 4/FGF-8
Nkx-3
Prop-1
Six-3
Brn-4
Isl1
P-ern
Shh
Ptx1
Lhx3, 4
Hesx1
BMP 2
Ptx1
Tpit
Prop1
Pit-1
POMC
NeuroD1
LIF
ACTH
(corticotrope)
TSH
(Pit-1-ind.)
thyrotrope
Ash1(?)
α-MSH
(melanotrope)
GATA2
SF1
DAX1
LH/FSH
gonadotrope
TSH
thyrotrope
GH
somatotrope
PRL
lactotrope

Pituitary gland development is dependent on the presence of both neuroectodermal and oroectodermal tissues of the same stage of development.[57,248] The signaling molecules and transcription factors involved in this process have been subject of extensive experimental investigation during the last decade. Each ectodermal layer produces signaling molecules resulting in an environment with temporary and spatial concentration-gradients in which the embryonic pituitary cells are stimulated to develop and to differentiate further (Figure 11).[60,65,76,227,248,258,259]

The hormone producing cells of the anterior lobe differentiate in a distinct temporal fashion (Figure 11).[226,227] The adenohypophyseal cells follow three main pathways of differentiation in the adenohypophysis; 1) the POMC-expressing cells (corticotrophs and melanotrophs), 2) the gonadotrophs and 3) the Pit-1 dependent cell lines (somatotrophs, lactotrophs and thyrotrophs).

2.3.1 The POMC-expressing cells

In the dog, the appearance of ACTH-immunoreactive cells in the adenohypophysis co-occurs with the in-growth of blood capillaries by e30.[226] The ACTH-immunoreactive cells are the first cells to be immunolabeled in the fetal dog adenohypophysis, similar to other species (Table 2).[44,55,226,227] There is also evidence that the fetal epithelium of Rathke's pouch in rats has the capacity for autodifferentiation into ACTH-producing cells.[139]

Overexpression of the fibroblast growth factor (FGF) 8 and leukemia inhibiting factor (LIF) favour the corticotroph cell lineage of the pituitary.[1,2,76,258,291]

Homeodomain transcription factors Ptx1 (also known as P-Otx1)[66,85] and Tpit expression are important for the development of POMC-expressing cells. During embryogenesis, Tpit is expressed at time of corticotroph differentiation (e12.5) in mice embryos and is required for late differentiation of corticotrophs and melanotrophs, but not for their cellular commitment.[141,212] NeuroD1 is expressed starting at e12.0 and contributes to differentiation of the corticotrophs. NeuroD1 deficient mice show a delayed differentiation of corticotrophs.[207,208]

Table 2
The approximate time points at which pituitary structures develop in different species[226,227]

	Dog (d)	Mouse (d)	Rat (d)	Pig (d)	Human (w)
Rudimentary RP	25	9	11	17	3
Definitive closed RP	27	11-12.5	12-14	22	≈5
ACTH	30	12.5	13.5	30	5
α-MSH	38				
α-GSU	na	11.5	11.5	≤50	<13
FSH, LH	38	16.5	16.5	50	12
TSH	na	14.5	14.5	40	9
GH	38	15.5	17.5	45	8
PRL	na	15.5	17.5	80	≤13
Full-gestation time (term)	63	20	22	114	40

D = days of gestation; na = not analyzed; w = weeks of gestation; RP = Rathke's pouch

2.3.2 The gonadotrophs

The differentiation of the bihormonal gonadotrophs is dependent on the transcription factors GATA-2 (NF-E1b) and SF1 (Ad4BP, Nr5al, FtzF1) for activation of gonadotroph-specific genes and terminal differentiation.[61,241,295] The Lhx gene family, particularly Lhx4 is also of importance for the development of the gonadotroph cell lineage.[186]

2.3.3 The Pit-1 dependent cells

The somatotroph, lactotroph and thyrotroph cell lineages are dependent on the expression and translation of Pit-1 (GHF-1) and Prophet of Pit-1 (Prop-1) for development, survival and hormone expression.[186,237,238] Prop-1 stimulates the Pit-1 gene expression,[238] which also is dependent on Lhx3 for its expression.[235]

2.3.4 Dual actions of differentiation factors

The factors that are involved in pituitary organogenesis may in addition to a growth and differentiation but also act as inhibitors of certain pathways. For example, the expression of Tpit seems to act as a regulatory switch between the POMC-producing cells and gonadotrophs. This was shown in Tpit deficient mice in which the cells of the intermediate lobe differentiated into gonadotrophs instead of melanotrophs.[212] Overexpression of LIF in the pituitary gland of transgenic mice resulted in corticotroph hyperplasia and repression of the other hormone-producing cell-lineages.[1,2] GATA-2 and Pit-1 have mutually antagonistic roles for development in the gonadotroph and thyrotroph cell ineages. High levels of GATA-2 in the presumptive gonadotrophs may inhibit Pit-1 expression. In the thyrotrophs, Pit-1 and GATA-2 are co-expressed at lower levels and are both important for activation of thyrotroph-specific genes.[61] Pit-1 may have a negative role in thyrotrophs, where it prevents GATA-2 binding to gonadotroph-specific promoters.[61]

2.3.5 Pituitary dwarfism in German shepherd dogs

Pituitary dwarfism in German shepherd dogs is an autosomal recessive inherited abnormality which is characterized by combined pituitary hormone deficiency (CPHD) and intrapituitary cyst formation.[133] Candidate genes among early transcription factors for pituitary development have been analyzed. In human and mouse, mutations associated with dwarfism are identified in Pit-1,[34,155,199,202,213] Prop-1,[68,82,203,238] Lhx4,[155] Lhx3 and Pitx2.[85,155,235] Also, overexpression of LIF in transgenic mice results in a distinctly short stature.[291]

Linkage analysis for dwarfism in German shepherd dogs has been done for Pit-1, Prop-1 and Lhx4.[145,146,269] Also, Pit-1 and Prop-1 are screened for mutations.[145,146] No co-segregation or mutation has been found, and the underlying defect remains to be discovered.

2.4 Pituitary-dependent hyperadrenocorticism, Cushing's disease

2.4.1 Hyperadrenocorticism

Hyperadrenocorticism, is one of the most common endocrinopathies in dogs, with a yearly incidence of about 1 per 1000 dogs.[217,285] Hyperadrenocorticism, also called Cushing's syndrome, was first described in humans in 1932 by the neurosurgeon Harvey Cushing. In dogs, hyperadrenocorticism was first described in 1939[275] and later in 1953, in 15 dogs by Coffin and Munson.[51] Hyperadrenocorticism can be caused by exogenous administration of glucocorticoids or by endogenous or spontaneous dysregulation of cortisol secretion. Endogenous hyperadrenocorticism is subdivided in ACTH-dependent (85%) and ACTH-independent (15%) hyperadrenocorticism.[216] Most cases of ACTH-dependent hyperadrenocorticism are of pituitary origin, caused by a corticotroph adenoma, but recently ACTH-dependent hyperadrenocorticism due to ectopic ACTH production has been described in the dog.[87] ACTH-independent hyperadrenocorticism is caused by functional adrenal tumors (adenoma or carcinoma).[192,285] Coexistence of pituitary and adrenal tumors may occur.[94,192]

2.4.2 Pituitary-dependent hyperadrenocorticism

Dogs with PDH are predominantly middle-aged, small breed dogs. Poodles, Dachshunds and Terriers are among the most commonly represented breeds.[216] Clinical signs can be ascribed to the underlying pathologic entities; hypercortisolism, hyperandrogenism, and expansion of the pituitary tumor. Glucocorticoids have glucogenetic, lipolytic, protein catabolic, anti-inflammatory and immunosuppressive effects. Persistent exposure is detrimental for the body. Hypercortisolism accounts for the most common and prominent signs of PDH, including polydipsia, polyuria, polyphagia, abdominal enlargement, lethargy, truncal obesity, skin changes (thin haircoat, bilateral symmetrical alopecia, comedones, pyodermia, calcinosis cutis), muscle weakness and decreased exercise intolerance (Figure 12). Hyperandrogenism may lead to perianal adenomas and mild polycytemia in bithces.[80] Due to mass effect of the pituitary tumor, signs may be seen such as behavioral changes, stupor, lethargy, anorexia, disorientation and ataxia.

One of the functional hallmarks of corticotroph adenomas is that they are less sensitive to the suppressive feedback effect of glucocorticoids. This explains why there is hypersecretion of ACTH in the face of elevated circulating glucocorticoid levels. Still many corticotroph adenomas are sensitive to high-dose dexamethasone administration which is used in the diagnostics of the disease. The diagnosis of hyperadrenocorticism is based upon the averaged urinary-corticoid-to-creatinine ratio (UCCR) in two consecutive morning urine samples combined with a high-dose dexamethasone suppression test.[219,242] After collection of the second urine sample, three oral doses of 0.1 mg dexamethasone per kg body weight are administered at 8-h 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 in the first 2 samples, the dog is categorized as being responsive to dexamethasone suppression and PDH is diagnosed.[86] In dogs with less than 50% suppression of the UCCR in the third sample, dexamethasone-resistent PDH can be confirmed by pituitary imaging, measurements of plasma adrenocorticotropic hormone (ACTH) concentrations, and visualization of the adrenals by ultrasonography.[29,218,267,278]

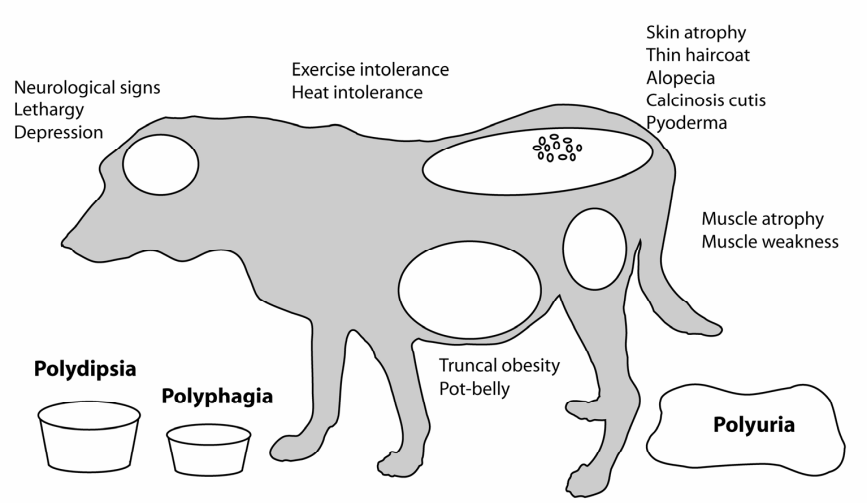


Figure 12. Schematic drawing of a dog with pituitary-dependent hyperadrenocorticism presenting clinical signs of hypercortisolism (Cushing's syndrome).

Corticotroph tumors are common in the dog, in contrast to the situation in humans.[72,167] However, sporadic GH secreting adenomas and non-functioning adenomas occur.[40,83,268] Nodules of focal hyperplasias and microadenomas are a common finding in the adenohypophysis of older dogs.[40] The pituitary adenoma can originate from the *pars distalis adenohypophysis* or in the *pars intermedia adenohypophysis* (Figure 13,14).[40]

It is unlikely that the canine corticotroph adenomas results from a chronic stimulation of CRH.[271] Partial mutation analysis of the Gsα, H-ras, K-ras, N-ras genes and the DNA-binding domain of the glucocorticoid receptor in 16 dogs with PDH revealed no mutations.[270] Thus, the underlying pathogenesis remains unknown.

2.4.3 Pituitary imaging

Pituitary imaging is an adjunctive diagnostic tool for pituitary tumors and enables visualization of the pituitary gland in the relation to osseous landmarks for transsphenoidal hypophysectomy.[176] For surgical planning, the thickness of the sphenoid bone can be measured. Because pituitary tumors in dogs do not erode bone or invade the sphenoid bone, survey radiographs of the skull are not helpful in the diagnosis.[170] Cisternography combined with linear tomography was initially used to indirectly visualize the pituitary gland[277] but was replaced by direct visualization of the pituitary gland by computed tomography (CT)[263,277,279] or magnetic resonance imaging (MRI).[266,267] The pituitary gland lies outside the blood-brain barrier[109] and contrast medium diffuses freely from the vascular to the interstitial space. Following intravenous administration of contrast medium the pituitary gland appears hyperdense as compared to brain tissue on contrast-enhanced CT images. The height and

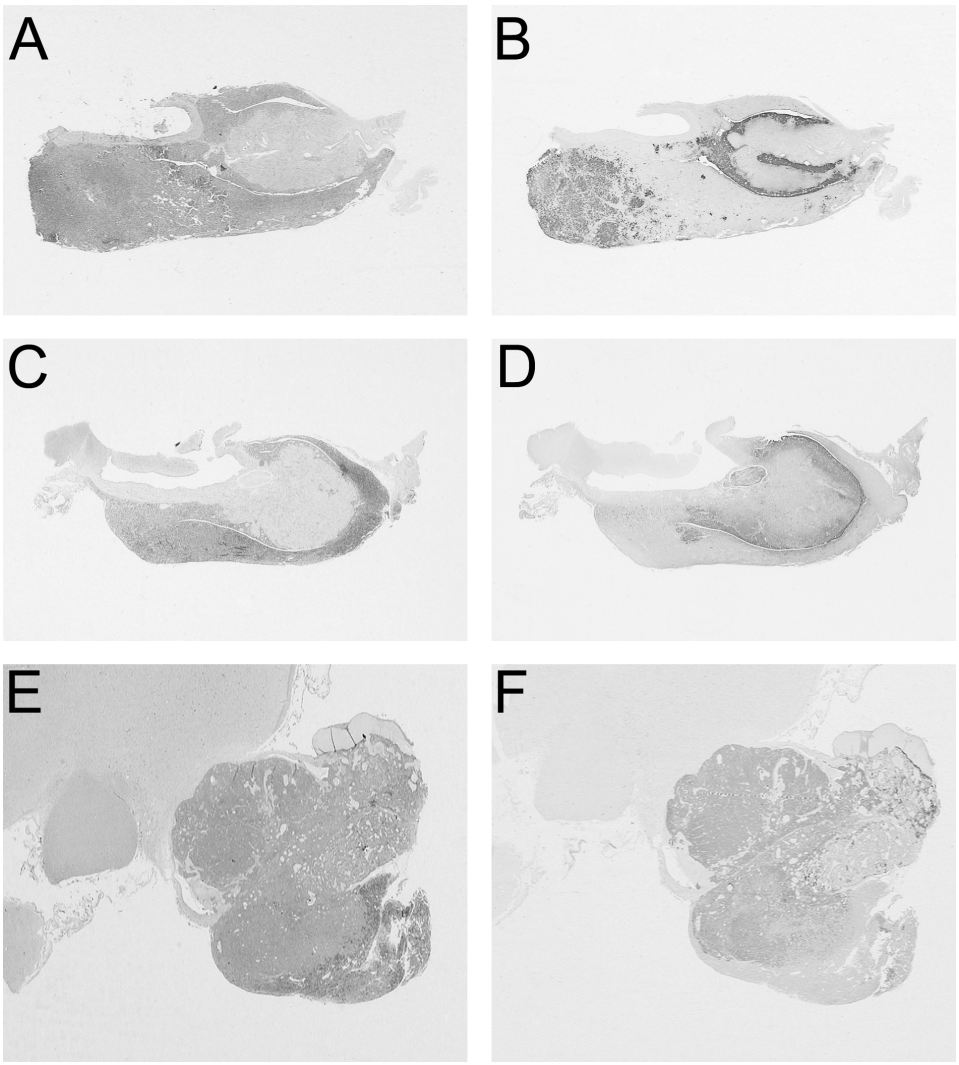


Figure 13. Histological (HE) stainings (A, C, E) and immunohistological stainings with anti-$ACTH_{1-24}$ (B, D, F) of sagittal sections of canine pituitaries; (A, B) Nodular hyperplasia/corticotroph adenoma in a pituitary of an old dog without clinical signs of hyperadrenocorticism (incidentaloma); (C, D) Pituitary microadenoma of a dog with pituitary-dependent hyperadrenocorticism; (E, F) Infiltrative macroadenoma (1.5 cm) with possible origin in the *pars intermedia adenohypophysis* (see color section).

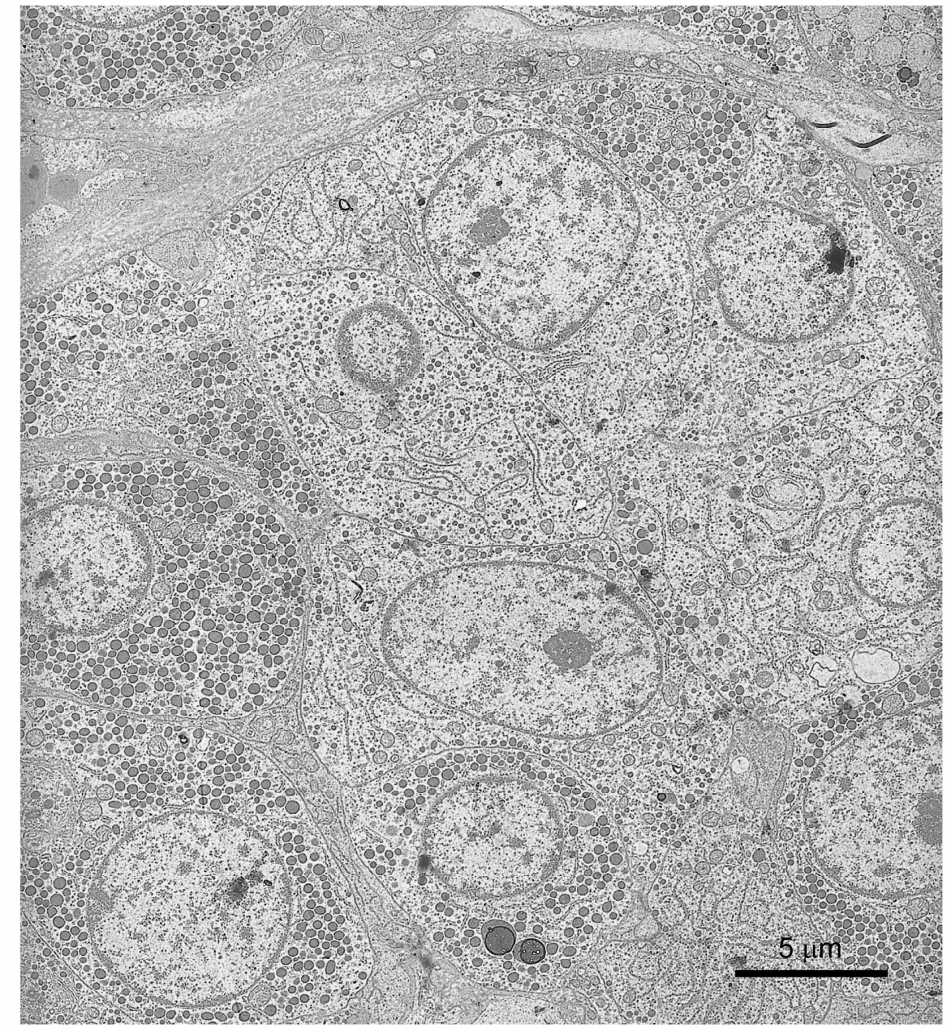


Figure 14. Electron microscopical photograph of a tissue specimen from an infiltrative pituitary adenoma, that was surgically removed by transsphenoidal hypophysectomy from a dog with pituitary-dependent hyperadrenocorticism.

width of the gland are measured on transverse images, and the length is measured from the sagittal image. Measurement of normal pituitary size on CT images underestimates the actual size. Mean pituitary sizes in 35 healthy dogs (29 beagles and 6 cross-breed dogs) on CT images measured 4.4 mm in height, 6.0 mm in width and 5.7 mm in length.[265]

Due to its high soft-tissue contrast, MRI is the preferred imaging technique for visualization of the pituitary gland in humans. In the dog, MRI visualizes the intracranial relation between the pituitary gland and the bordering bony structures, the tuberculum sellae and the dorsum sellae, especially on the sagittal slices.[266] On MRI, the normal mean ± S.D. pituitary height is 5.1 ± 0.9 mm and width is 6.4 ± 1.1 mm.[127] To distinguish enlarged from nonenlarged pituitary glands in dogs with varying skull sizes and shapes, the pituitary height-to-brain-area ratio (P/B ratio) is calculated.[134] Pituitary glands with a P/B ratio >0.31 (x 10^{-2} mm^{-1}) are considered enlarged and those with a P/B ratio ≤0.31 (x 10^{-2} mm^{-1}) nonenlarged. In approximately 40% of the dogs with PDH, the size and shape of the pituitary gland is not changed.[134] Additionally, for surgical planning, the thickness of the sphenoid bone is measured.

Enlarged pituitaries are readily diagnosed on CT and MR images.[67,134] In dogs with nonenlarged pituitaries, microadenomas are usually difficult to distinguish from non-neoplastic pituitary tissue because of isoattenuation.[263] The adenoma may be indirectly visualized by the displacement or distortion of the 'pituitary flush' on contrast-enhanced dynamic CT images.[263] The pituitary flush represents the contrast enhancement of the central vasculature of the *neurohypophysis*, which follows the enhancement of the internal carotid arteries in the cavernous sinuses, and preceeds the peripheral enhancement of the secondary capillary bed of the *pars distalis adenohypophysis.*[160,264] A displacement of the pituitary flush indirectly indicates the position of the adenoma and agrees to a high extent, but not completely, with surgical and histopathological findings.[263,267] Absence of the pituitary flush indicates a diffusely abnormal pituitary gland.[263] Further advancement in pituitary imaging is to be expected. Dynamic helical CT may give insight in the three-dimensional characteristics of the neurohypophyseal flush, and indirectly may give insight in the three-dimensional location of the adenoma.[262] MRI may allow direct observation of pituitary (micro) adenomas when they reveal themselves as hypodense compared to more hyperdense normal pituitary tissue. Adequate direct visualization of the pituitary (micro) adenomas with MRI depends on slice thickness and the magnet strength of the scanner used.

2.4.4 Treatment of PDH

Treatment for PDH in dogs can be medical (e.g., administration of an adrenocorticolytic agent or an inhibitor of cortisol synthesis), radiotheapeutic, or surgical (bilateral adrenalectomy or transsphenoidal hypophysectomy).

2.4.4.1 Medical treatment

Because of technical problems with pituitary surgery, most treatment regimes in dogs with PDH are not directed to the underlying pathology (pituitary adenoma) but to the adrenal gland where a reduction of cortisol production is aimed at. Chemotherapy with mitotane or o,p'-DDD has most commonly been used for managing dogs with PDH.[64,126] Mitotane causes progressive necrosis of the zona fasciculata and zona reticularis of the adrenal cortex, inflicting irreversible changes to the adrenal gland.[80,190] More recently, trilostane was introduced.[17,22,189,222,236,288] Trilostane is a competitive inhibitor of 3β-hydroxysteroid

dehydrogenase, an essential enzyme for the synthesis of cortisol, aldosterone and androstenedione.[206,236] Trilostane treatment results in a reduction of plasma cortisol for part of the day enough to relieve clinical signs. However, plasma ACTH concentration increases.[236] Although the intrinsically elevated glucocorticoid exposure does not prevent the progression or proliferation of the corticotrophic tumor cells, it may be hypothesized that the elevated glucocorticoid levels in dogs with PDH may slow down the proliferation of the corticotroph adenoma, parallel to findings *in vitro*.[271] Directing the treatment to the adrenal cortex and cortisol synthesis has a theoretical risk of inducing a higher growth rate of the pituitary adenoma due to the elimination of the cortisol feed-back inhibition. A similar mechanism is reported in human patients with Cushing's disease who develop Nelsons's syndrome after bilateral adrenalectomy. Nelson's disease is characterized by markedly elevated serum concentrations of ACTH and related POMC peptides, and a rapid and aggressive growth and transformation of the pituitary adenoma into an invasive pituitary adenoma.[122,260] So far it is known that trilostane therapy results in significantly increased plasma ACTH,[236] and that mitotane treatment of healthy dogs leads to corticotroph hyperplasia.[251]

2.4.4.2 Radiotherapy

There is limited experience with radiotherapy for dogs with PDH. Initial decrease in tumor size has been reported but recurrences are common.[93,257]

2.4.4.3 Surgical treatment

Adrenalectomy is the treatment of choice for adrenocortical adenomas and carcinomas in dogs,[7,228] but not the treatment of choice for PDH. In humans, adrenalectomy is associated with an increased risk of developing Nelson's syndrome.[122,260]

In humans with Cushing's disease, pituitary surgery and selective adenomectomy of the pituitary adenoma through the transsphenoidal route is the treatment of choice.[18,150] The transsphenoidal route was first described in humans by Schloffer and successfully practiced by the neurosurgeon Harvey Cushing, which also gave name to the syndrome of hyperadrenocorticism (Cushing's syndrome) and pituitary-dependent hyperadrenocorticism (Cushing's disease).[169]

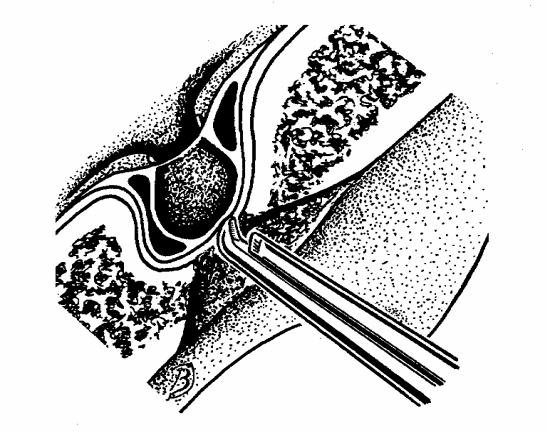


Figure 15. Transsphenoidal hypophysectomy. Midsagittal cross-section through the sphenoid bone and the pituitary fossa. A hole has been burred in the sphenoid bone and the inner cortical lamina of the sphenoid bone is removed with a 1 mm bone punch (With permission, Meij 1997).

Transsphenoidal hypophysectomy in experimental dogs was described in detail in 1956,[163] and applied to dogs with PDH in 1977 by Lubberink.[161] However, the lack of preoperative localization of the pituitary gland in brachycephalic dogs and dogs with small skulls limited its use as a preferred technique. With the emergence of new imaging techniques (CT and MRI), accurate assessment of pituitary size and localization of the pituitary gland in the relation to the surgical landmarks was enabled. The technique was refined in Utrecht and proved to be effective for the treatment of dogs with pituitary-dependent hyperadrenocorticism.[176,177] The technique is a transoral, transnasopharyngeal, transsphenoidal microsurgical approach to the pituitary gland with the dog in sternal recumbency. The aim of pituitary surgery in the dog is to completely remove the pituitary gland including the adenoma, in other words a hypophysectomy (Figure 15). After hypophysectomy, hormone replacement therapy consists of lifelong cortisone acetate, thyroxine, and temporary administration of desmopressin, a synthetic vasopressin analogue. The major complications are postoperative mortality, hypernatremia due to acute AVP deficiency, prolonged diabetes insipidus (chronic AVP deficiency), keratoconjunctivitis sicca (KCS), residual disease and recurrence of hyperadrenocorticism. A recent edition of a well-known textbook on clinical endocrinology in dogs and cats stated "In our opinion, this procedure is the treatment of choice for dogs with PDH. The only explanation for not using this form of therapy as a 'routine' procedure is the lack of experienced neurosurgeons willing to develop this expertise".[80] Table 3 summarizes studies including hypophysectomy in dogs and cats.

Table 3
Studies including hypophysectomy (HX) in normal dogs, and in cats and dogs with pituitary-dependent hyperadrenocorticism (PDH)

Studies including hypophysectomy (HX) in period 1997-2006	Normal	PDH		Country	Year	Reference
	dog	dog	cat			
Surgical technique	8			NL	1997	Meij *et al.* [177]
Pituitary function after HX	8			NL	1997	Meij *et al.* [172]
Pituitary function after HX		39		NL	1997	Meij *et al.* [171]
Results in PDH		52		NL	1998	Meij *et al.* [176]
Results in PDH			7	NL	2001	Meij *et al.* [175]
Review on HX in PDH		84	7	NL	2002	Meij *et al.* [168]
Dynamic CT in PDH for HX		55		NL	2003	Vlugt-Meijer *et al.* [263]
Results in PDH		4		Japan	2003	Hara *et al.* [101]
Desmopressin and DI after HX	10			Japan	2003	Hara *et al.* [100]
CR: double pituitary adenoma			1	NL	2004	Meij *et al.*[174]
CR: melanotroph pituitary adenoma			1	NL	2005	Meij *et al.* [173]
CR: ectopic ACTH secretion after HX		1		NL	2005	Galac *et al.* [87]
Surgical technique	9			USA	2005	Axlund *et al.* [15]
Results in PDH		150		NL	2005	Hanson *et al.* [99]
Na+, K+-ATPase in skeletal muscle	6	13		NL	2006	Schotanus *et al.* [233]
Posterior lobe function after HX	14			Japan	2006	Taoda et al. [250]
6-h Plasma profiles after HX		17		NL	2006	Hanson *et al.* [98]

CR = case report; NL = Netherlands; USA = United States of America; DI = diabetes insipidus; PDH = pituitary-dependent hyperadrenocorticism

2.5 Pituitary tumors in humans

2.5.1 Tumor classification and epidemiology (humans)

Anterior pituitary tumors are commonly encountered lesions that account for 7-15% of all primary intracranial neoplasms, being the third most common intracranial neoplasm after meningiomas and gliomas.[43] Based on their size, pituitary adenomas are traditionally divided into microadenomas (<10 mm) and macroadenomas (>10 mm).[77] The prevalence of pituitary tumors in autopsy and radiologic studies is relatively high (10-20%), the large majority comprising microadenomas.[38,77,185] The reported annual incidence of clinically relevant pituitary adenomas varies between 8.2-14.7 per 100 000 population.[243,253] Recently a high prevalence of clinically relevant tumors of 94±19.3 cases/100 000 population was reported from Belgium.[59]

Of the diagnosed pituitary adenomas, between 6-10% are ACTH-producing (corticotroph adenomas)[59,253] (Table 4). Corticotroph adenomas are predominantly seen in women with a female to male ratio ranging from 3:1 to 10:1.[182,253] Peak incidence for corticotroph adenomas is during the third and fourth decades of life.[182]

The prevalent WHO classification, based on the ICD codes available, divide pituitary tumors into 1) typical pituitary adenoma, 2) atypical pituitary adenoma and 3) pituitary carcinoma.[5] Pituitary tumors with invasive growth, elevated mitotic index, Ki-67 (MIB-1) labeling index (see below) are >3% and p53 nuclear immunoreactivity >3% considered atypical and pituitary tumors with distant metastasis are classified as carcinoma. This anatomical and histological classification does not differentiate between tumors of different pituitary cell lineages. Therefore, a new classification of the neoplasms is used based on immunoreactive and ultrastructural characteristics is being used by leading pathologists of the field (Table 4).[5,25]

Also the definition of pituitary carcinomas is being criticized. With the current definition, pituitary carcinomas are defined as pituitary tumors with subarachnoid, brain, or systemic metastasis.[231] Pituitary carcinomas are rare, constituting only 0.1-0.2% of pituitary tumors.[200] Most carcinomas are of predominantly ACTH or PRL secreting.[214] There is a need for malignant criteria for pituitary tumors that reflect their invasiveness and tendency for recurrence independently of the occurrence of metatstases.[5]

To elucidate the pathogenesis and to find markers for prognostic outcome, much effort is put into the classifications of human pituitary tumors, which will be reviewed below.

2.5.2 Clonality as argument for the origin of the pituitary adenoma

To determine whether the pituitary adenomas are secondary sequels to a primary disease elsewhere or a primary pituitary disease, chromosomal analyses have been used to determine the clonality of the tumor. Monoclonality was considered as a proof of a tumor origin from one cell only, thus evidence for a primary pituitary disease. Based on allelotype analysis[292] and X chromosome inactivation analysis in women[26,91,102,234] the majority of human ACTH-secreting pituitary adenomas (27 out of 31) are monoclonal.[12,26,91,102,154,234] Therefore, the pituitary tumors have been considered to originate from a single pituitary cell with somatic mutation(s). However, monoclonality does not exclude the presence of trophic responses to stromal or environmental signals.[49,154] Initial and recurrent tumors of a benign tumor type are frequently derived from separate independent clones. This suggests either the existence of more than one abnormal clone initially, of which one will become the dominating part, or

Table 4
Developmental lineage classification and taxonomy of neoplasms. Classification of pituitary adenomas in humans by cytodifferentiation and their relative frequency.

Adenoma type	Transcription factors	Immunoreactivity and histological characteristics	Rel. freq. (%)	Micro-adenoma		Macro-adenoma		Overall incidence of invasion
				%	Inv%	%	Inv%	
The Pit-1 family								
Somatotroph (S)				14	0	86	50	50
Densely granulated S.	Pit-1	GH, α-Subunit	7.1					
Sparsely granulated S	Pit-1	Keratin whorls (fibrous bodies)	6.2					
Mammosomatotroph/ mixed	Pit-1, ER	GH, PRL, α-Subunit	4.7	26	0	74	31	31
Lactotroph (L)				33		67		52
Sparsely granulated L	Pit-1, ER, ?GH-repressor	PRL, Golgi pattern	27					
Densely granulated L	Pit-1, ER, ?GH-repressor	PRL diffuse cytoplasmatic	0.04					
Acidofil stem cell	Pit-1, ER	PRL, (GH), keratin whorls (fibrous bodies)	1.6					
Thyrotroph	Pit-1, TEF, GATA-2	β-TSH, α-subunit	1.1	0	0	100	75	75
Plurihormonal	Pit-1, ER, TEF, GATA-2	GH, PRL, β-TSH, α-subunit						
ACTH family								
(Densely granulated) corticotroph (C)	Tpit	ACTH, keratins	9.6	87	8	13	62	15
Silent 'C' adenoma subtype 1		ACTH	1.5					
Silent 'C' adenoma subtype 2		Beta-endorphin, ACTH	2.0					
Gonadotropin family	SF-1, ER, GATA-2	β-TSH, β-LH, α-subunit						
Gonadotroph			9.8	0	0	100	21	21
Unclassified adenomas								
Hormone-negative/ null cell	None	None	12.4					
Unusual plurihormonal		Multiple	1.8					

ER = estrogen receptor; GH = growth hormone; PRL = prolactin; TEF = thyrotroph embryonic factor; TSH = thyroid-stimulating hormone; ACTH = adrenocorticotropic hormone; FSH = follicle-stimulation homone; LH = luteinizing hormone; SF = steroidogeneic factor; Inv = invasion. Rel. freq. = Relative frequences (derived from Russel and Rubenstein [253])

that several clones arise independently at different times. In conclusion, monoclonality does not exclude the existence of a exogenous stimulation.[50]

2.5.3 Pituitary proliferation and markers therefore

The pituitary gland is a dynamic tissue in which cells proliferate upon stimulation, e.g., in response to an increased physiological need. The most mitotic activity occurs in cells that are not immunopositive for any of the pituitary hormones.[152] There is a high turnover rate of cells in the pituitary that decreases with age.[152,249] A general reduction in pituitary cell turnover with age has been noted in beagle dogs.[152] In rats a circadian rhythm in mitotic activity has been suggested.[152] CRH has a trophic potential on the pituitary[46,152] and chronic administration has been associated with increased numbers of corticotrophs,[13,90] corticotroph nodular hyperplasia (in untreated Addision's disease),[230] and in a single case report of an intrasellar CRH producing gangliocytoma with corticotroph adenoma formation.[224] Vasopressin is a more potent inducer of mitotic activity than CRH in rat anterior pituitary cells. Administered together, however, low levels of CRH enhance low-dose AVP induced proliferation.[166]

There are several ways of estimating the pituitary proliferation, of which the Ki67 labelling index may be the most useful. The Ki67 antigen (MIB-1) is a protein related to cell proliferation and is expressed in cell nuclei throughout the entire cell cycle except in resting cells (G0), and as such can be used as a marker for proliferative active cells, both normal and tumorous.[14,89,95,209,232,254,284] The Ki-67 is homogenously present in pituitary adenomas, the percentage of stained cells are mostly reported in the range 0.2-4.6%.[128,130] with exceptional cases with a labeling indices of 15-23%.[149,159,204,209] High Ki-67 labelling index has been associated with a higher growth rate and shorter tumor doubling time (range, 200-2550 days) in recurrent pituitary adenomas.[107] A higher Ki-67 labelling index has been found in macroadenomas (9.3%) than microadenomas (2.8%) in patients with Cushing's disease.[159] Pituitary adenomas with higher Ki-67 are more prone to present with dura mater invasion,[128,130] but there are contradicting studies published on Ki-67 and the correlation with and prediction of tumor invasiveness.[209] Pituitary carcinomas have significantly higher mean Ki-67 than pituitary adenomas, but the range is high (0-21.9%)[115,256] Ki-67 labelling index also correlates strongly with p53.[255,256]

The mitotic count is another way of estimating degree of proliferation. However, the mitotic count represents only a small proportion of the entire cell population's growth fraction.[209] The mitotic index is usually low.[214] With a preoperative injection of bromodeoxyuridine (BrdU) the proliferative capacity measured as the index of cells in DNA synthesis phase (S phase) can be measured in situ. Recurrent tumors have a higher LI (1.4% than primary tumors 0.3%.[214]

2.5.4 Corticotroph tumors – histopathologic and ultrastructural characterization

Pathologically, the corticotroph adenomas are of two types, the densely and sparsely granulated corticotroph adenomas.[253] The densely granulated corticotroph adenomas are the most common in humans, and appear as PAS-positive basophilic adenomas. Sparsely granulated corticotroph adenomas are PAS negative, chromophobic, and has an aggressive behavior and invasive tendency.[253] In humans there is a tendency for corticotroph adenomas to have an inverse relationship between the size of the adenoma and the level of plasma

ACTH concentrations and symtoms of cortisol excess. Microadenomas are more likely to be associated with more conspicuous clinical symtoms than the milder forms that the macroadenomas usually are associated with.[253] The corticotroph adenomas are positive for cytokeratin immunoreactivity for type 1 microfilaments. Plurihormonality is uncommon in corticotroph microadenomas, but a weak or focal immunoreactivity for LH and/or the α-subunit may be seen in some macroadenomas, particularly in recurrent cases.

The densely and sparsely granulated corticotroph adenomas are distinguished with electronmicroscopy. Densely granulated adenomas are composed of medium-sized angular cells, with oval nuclei, and well-developed reticular endoplasmatic reticulum (RER) and Golgi-complex. Granules (200-450 nm in diameter) are seen in abundance throughout the cytoplasm, but accumulating at the cell periphery below the cell membrane, and are of typical dented, heart or teardrop shape. This typical shape of the granules are one hallmark of the corticotroph adenomas, the other is the presence of perinuclear parallel bundles of cytokeratin microfilaments (type 1).[253] The sparsely granulated corticotroph adenomas are smaller, have poorly developed cytoplasmic organelles, and contain few, small sized secretory granules. The type 1 microfilament is sparse. In human corticotroph cells, presence of a state of hypercortisolism results in to massive perinuclar, ring-like accumulation of keratin filaments known as Crooke's hyalinization. This change is seen in the non-neoplastic corticotrophs but can occasionally occur in the adenomateous corticotrophs as well, but with no significance for clinical or prognostic significance.[167,253]

2.5.5 Molecular genetics in human corticotroph tumors

In contrast to the somatotropinomas, where 40% of the tumors carry mutations of the stimulatory α-subunit of the G protein complex[143,286] the underlying molecular pathogenesis of corticotroph adenomas in humans, remains unknown. Tumors can be regarded as a genetic disease of somatic cells, and oncogenesis a multistep process that can be divided into three (overlapping) phases; the initiation, which is a DNA damage in normal cell; the promotion, which is the outgrowth of pre-cancerous cells; and the progression which is the process of accumulation of additional genetic changes leading to increased malignancy. The genes that are damaged can be divided into two main categories; activation of protoncogenes converting them to oncogenes (including enhanced expression and/or altered product) and inactivation of tumor suppressor genes. The protooncogenes include growth factors and their receptors, signaling peptides downstream the growth-factor receptor and transcription factors. The tumor suppressor genes involve differentiation factors, factors controlling cell cycle, DNA repair and apoptosis[3,11].

2.5.6 Protooncogenes

Several protooncogenes (e.g., ras, G protein, myc) have been screened for mutations, with only a limited number of mutations found (Table 5).[12,56] However, with few exceptions only small numbers of corticotroph adenomas are included in the studies performed (Table 5).

At the protein level, a major finding was made by Bilodeau *et al* 2006 that about 50% of the human corticotroph tumors (and canine) lack two proteins (Brg1and HDAC2) that are involved in the glucocorticoid receptor comples.[27]

Table 5
Putative and studied protooncogenes in human corticotroph adenomas

Factor	Analysis	Tumor Material	Findings	*n* corticotroph adenomas Changed	Total	Ref
G-proteins	M-analysis Gsα (gsp) and Gi2α (gip)	gDNA	M	1	5 (NFT)	Williamson *et al.* 1994[286]
G-proteins	M-analysis Gsα (gsp) and Gi2α (gip)	gDNA	M	3	32	Williamson *et al.* 1995[287]
G-protein	M-analysis Gsα	gDNA	M	1	1	Riminucci *et al.* 2002[220]
Ras	Partial M- analysis codon 12, 13, 61	gDNA	NC	0	5 (C)	Cai *et al.* 1994[39]
Ras	Partial M-analysis codon 12, 13, 61	gDNA	NC	0	3 (C)	Pei *et al.* 1994[197]
			M (H-ras)	2	3 (me)	
Ras	Partial M-analysis codon 12, 13, 61	gDNA	NC	0	1 (NFT)	Karga *et al.* 1992[117]
Ras, myc, mycL1, mycN, bcl1, H-stf1, sea, kraS2, fos	Chromosomal amplification and rearrangement	gDNA	NC	0	≥2	Boggild *et al.* 1994[28]
RET	M-analysis exon 10, 11, 13, 15	gDNA	NC	0	1	Komminoth *et al.* 1996[131]
c-ERB2/neu	Semiq.E expression analysis	cDNA	NC	0	1	Ezzat *et al.* 1997[78]
	Partial M analysis	gDNA	NC	0	1	
c-myc	Imm. hist.	tissue	↑P	3	3	Ikeda & Yoshimoto 1992[111]
PKC	Western blot analysis	Protein	↑P	1	1	Alvaro *et al.* 1993[6]
	M analysis	cDNA	M	1	1	
PKC			↑A		1	Couldwell *et al.* 1996[53]
PTTG	Semiq. E analysis (RT-PCR)	cDNA	↑E	1	1	Zhang *et al.* 1999[293]
PTTG	M-analysis PTTG promotor	gDNA	NC	0	25	Kanakis *et al.* 2003[116]
PTTG, PBF,	Quantitative PCR	cDNA	(↑E PTTG, ns)		5	McCabe *et al.* 2003[165]
FGF-2, FGF-R-1			↑E KDR			
VEGF, KDR/Flk-1						McCabe *et al.* 2002[164]
Glucocorticoid receptor	M-analysis (Nelson's syndrome)	gDNA	M	1	4	Karl *et al.* 1996[119]
Glucocorticoid receptor	M-analysis	gDNA	M	1	1	Karl *et al.* 1996[118]
Glucocorticoid receptor	M-analysis	gDNA, cDNA	NC	0	18	Antonini *et al.*[10]
LIFR	M-analysis	cDNA	NC	0	7	Heutling *et al.* 2004[103]
Tbx19/Tpit	M-analysis	cDNA	NC	0	8	Bucciarelli *et al.* 2005[36]
Brg1	Imm. Hist.	tissue	↓P, Δ P	12	36	Bilodeau *et al.* 2006[27]
HDAC2			↓P	5	36	

↑E = increased gene expression; ↓E = reduced gene expression; Δ E = change from nuclear to cytoplasmic location; ↑A = increased activity; ↑P = protein accumulation; ↓P = reduction/loss in protein; NC = no change; LOH = loss of heterozygosity; (h) = in humans; M = mutation; me = metastasis; Ad = Adenomas; Ag = aggressive tumors; C = carcinomas; cDNA = copy DNA; gDNA = genomic DNA; FGF = Fibroblast growth factor; I = invasive tumors; KDR/Flk-1 = VEGF receptor; NFT = non-functioning

LIF may act as a protoncogene by stimulating the corticotroph cells.[1,2,144] By binding to the LIFR intracellular signalling pathways are activated including STAT3, the oncogenetic role of which has been discussed.[33]

The importance of proper silencing of factors involved in normal pituitary development is exemplified by the development of adenomatous hyperplasia with formation of Rathke's cysts and tumors in aged transgenic mice with constitutive expression of Prop-1.[54]

2.5.7 Tumor suppressor genes

Among the tumor suppressor genes are involved in carcinogeneses are the cyclins, cyklin-dependent kinases (cdk) and inhibitors of this system (p53, p27), purine-binding factor (nm23), the retinoblastoma gene (Rb), the multiple endocrine neoplasia (MEN) gene 1, and genes that are regulating apoptosis (GADD45γ, Zac). The tumor suppressor p53 is a nuclear protein which regulates the gene expression of the cdk $p21^{Cip1/Waf112}$ and among the most commonly mutated genes in human tumors.[105,151] An abnormal, increase in p53 immunostaining has been observed in corticotroph adenomas. The immunolabelling increases with invasiveness. It is high in carcinomas and even higher in the metastastatic tissue.[37,84,229,255] But so far no mutation in corticotroph adenomas has been found.[153,197]

Another factor studied is the inhibitor of the cyclin-cdk complex p27 kip1. Deficiency leads to predisposition for selective hyperplasia of the intermediate lobe of mice and the development of POMC positive pituitary tumors in a form of inheritable multiple endocrine neoplasias (MEN-1).[81,129,188,198] A generally low expression of p27 in normal corticotrophs and corticotroph adenomas was found in one study,[156] but not confirmed by another.[229] However there was a wide range of immunopositive cells among the tumors,[229] and a normal degree of phosphorylated P-p27 has been demonstrated despite a low total immunopositivety for p27.[135]

Cyklin E is essential for cell-cycle regulation and may act as an oncogene and may be upregulated by loss of regulatory control by p53.[19,113] Overexpression is associated with poorly differentiated and invasive hepatocellular carcinoma and is also found in breast carcinomas.[110] Corticotroph adenomas have a higher expression of cyclin E than other pituitary adenomas.[113,144,156]

Reduced levels of the purine-binding and metastasizing suppressor gene nm23 have been found in breast cancer and lymph node metastases and metastastasizing hepatocellular carcinoma and colorectal cancer. In one study there was an association with the levels of nm23 expression and invasiveness into cavernous sinus, among which one corticotroph adenoma was included.[247]

Loss of Rb in transgenic mice is highly associated with the development of tumors of the intermediate lobe with concurrent high plasma concentrations of α-MSH.[108] Loss of Rb expression in an ACTH-secreting carcinoma[104] and loss of heterozygosity has been reported for corticotroph adenomas.[195,196,289]

The menin (MEN1) gene is associated with inherited forms of multiple endocrine neoplasias, which includes corticotroph adenomas.[12]

GADD45γ also known as cytokine response gene (CR6) is DNA-damage inducible gene that is involved in growth-arrest and apoptotic pathways.[246,280] GADD45γ is a strong repressor of colony formation of cell lines e.g., AtT20., and reduced or loss of expression is seen in non-functional and GH and PRL secreting pituitary tumors.[16,294]

A novel apoptosis gene, PTAG (pituitary tumor apoptosis gene), is found to be metylated in pituitary tumors. Overexpression of PTAG in AtT20 cells increases the sensitivity to apoptopic effects.[79]

2.5.8 Genomic instability

An increased genomic instability and chromosomal aberrations have been observed in pituitary carcinomas and metastases and with an inceased frequency in functioning, invasive and recurrent adenomas.[229,244] A factor that has been associated with an increased chromosomal instablility is the pituitary tumor transforming gene (PTTG).[45,180]

2.5.9 Pituitary adenomas expressing more than one hormone

In humans it is not uncommon with pituitary tumors that express more than one hormone. Usually, such tumors represent one cell lineage. There are however, also described patients with composite adenomas, co-expressing hormones of different lineages e.g., ACTH and GH. In several of those, there is a distinction between GH and ACTH producing cells but there are also cases with proven co-expression of both GH and ACTH in the same tumor cells. [245]

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PART I

Pathobiology of pituitary function after transsphenoidal hypophysectomy in dogs with pituitary-dependent hyperadrenocorticism

CHAPTER 3

Efficacy of transsphenoidal hypophysectomy in treatment of dogs with pituitary-dependent hyperadrenocorticism

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Abstract

The long-term survival, disease-free fractions, and the complications of hypophysectomy in 150 dogs with pituitary-dependent hyperadrenocorticism (PDH) were examined in a prospective study. Long-term survival and disease-free fractions in relation to pituitary size were analyzed by the Kaplan-Meijer estimate procedure.

The 1-, 2-, 3-, and 4-year estimated survival rates were 84% (95% confidence interval [CI], 76-89%), 76% (67-83%), 72% (62-79%), and 68% (55-77%), respectively. Treatment failures included procedure-related mortalities (12 dogs) and incomplete hypophysectomies (9 dogs). The 1-, 2-, 3-, and 4-year estimated relapse-free fractions were 88% (CI: 80-93%), 75% (64-83%), 66% (54-76%), and 58% (45-70%), respectively. Postoperative reduction of tear production (58 eyes in 47 dogs) was often reversible but remained low until death in 11 eyes of 10 dogs. Central diabetes insipidus (CDI) occurred more frequently (62%) in dogs with enlarged pituitaries than in dogs with nonenlarged pituitaries (44%). Survival and disease-free fractions after hypophysectomy were markedly higher in dogs with nonenlarged pituitaries than in dogs with enlarged pituitaries.

Transsphenoidal hypophysectomy is an effective treatment for PDH in dogs. The survival and disease-free fractions after hypophysectomy decrease, and the incidence of CDI increases with increasing pituitary size. Therefore, early diagnosis of PDH is important and transsphenoidal hypophysectomy is expected to have the best outcome when used as primary treatment for dogs with nonenlarged or moderately enlarged pituitaries.

Hyperadrenocorticism, or Cushing's syndrome, is the complex of physical and biochemical changes resulting from chronic exposure to glucocorticoid excess. In pituitary-dependent hyperadrenocorticism (PDH), excess ACTH is produced by pituitary corticotropic adenomas that may range in size from microadenomas to large tumors.[16,25] The most common treatment in dogs has been the selective or non-selective elimination of the glucocorticoid excess by chemotherapy with the adrenocorticolytic drug o,p'DDD (mitotane), which has a relative high recurrence rate.[7,15] Recently, treatment with a competitive inhibitor of adrenal 3β-hydroxysteroid dehydrogenase has been introduced.[6,24,30,38] Results of long-term follow-up are not yet available.

Although effective, these treatments are not directed at the elimination of the primary lesion, the ACTH-producing pituitary adenoma. The pituitary tumor may continue to expand, leading to neurological signs due to an intracranial mass effect.[2,8] Pituitary-tumor growth may even be promoted by treatment that eliminates the suppressive negative feedback of glucocorticoids on pituitary tumor growth. Bilateral adrenalectomy in humans with Cushing's disease may result in markedly high ACTH concentrations, and aggressive invasive pituitary-tumor growth.[14] There are few treatment options once a pituitary macroadenoma has resulted in neurological signs.

Early diagnosis, pituitary imaging, and treatment at the pituitary level should be the hallmarks of a treatment protocol for canine PDH. Selective pituitary microsurgical adenomectomy by the transsphenoidal approach is considered the treatment of choice for pituitary tumors of the sellar region causing Cushing's disease in humans.[26] In 1993, in Utrecht, The Netherlands, hypophysectomy was reintroduced as treatment of PDH in dogs. Short-term (≤3 years) results were reported in a group of 52 dogs,[21] and since then, the study has progressed.[19] In the present study long-term survival, disease-free fraction, complications and the relation between pituitary size and the long-term results in 150 dogs with PDH are described.

Materials and Methods

Animals

One hundred and fifty dogs with PDH, referred to the Utrecht University Clinic for Companion Animals, over a 10-year (1993–2003) period underwent transsphenoidal hypophysectomy. Crossbred dogs and purebred dogs were represented (Table 1). Head shapes varied from brachycephalic (eg, French bulldog, Dogue de Bordeaux) to mesaticephalic (the majority of the dogs) to dolichocephapic (eg, collie type). Sixty-eight dogs were male and 82 were female. The age at the time of surgery ranged from 3 to 14 years (median 9 years), and the body weight of the dogs ranged from 4 to 61 kg (median 15 kg).

Apart from the characteristic clinical signs, such as polyuria, skin atrophy, and increased abdominal size, the diagnosis of hyperadrenocorticism was based upon biochemical changes[27] and high ($\geq 10 \times 10^{-6}$) urinary corticoid-to-creatinine ratios (UCCR) measured in 2 consecutive morning urine samples collected by the owner.[29,33] Immediately after collection of the 2nd urine sample, the animals received 3 doses of 0.1 mg dexamethasone/kg PO at 8-hour intervals. The next morning, a 3rd urine sample was collected. When the UCCR in the 3rd sample was less than 50% of the mean in the 1st 2 samples, the dog was categorized as being responsive to dexamethasone suppression, and PDH was diagnosed. In 123 dogs, the

combination of high UCCRs and dexamethasone suppression allowed the diagnosis of PDH. In 27 dogs, there was resistance to dexamethasone suppression and pituitary-dependency was demonstrated by measurements of plasma-ACTH concentrations and further supported by visualization of the adrenals by ultrasonography and pituitary imaging.[5,28,37] In 149 dogs, the basal UCCR exceeded the upper limit of the reference range, ranging from 10 x 10^{-6} to 598 x 10^{-6}. In 1 dog UCCR was not available. Diagnosis was made at another institution based on ACTH stimulation and the low-dose dexamethasone suppression test.

Table 1
Breeds of 150 dogs with pituitary-dependent hyperadrenocorticism

Breed	No.
Wire-Haired Dachshund	12
Minature Poodle	11
Jack Russell Terrier	6
Maltese	6
Yorkshire Terrier	5
English Cocker Spaniel	4
Labrador Retriever	4
Beagle	3
Bouvier des Flandres	3
Dalmatian	3
German Shepherd	3
Medium-sized Poodle	3
Crossbred	31
Other breeds	56

Pituitary imaging

The pituitary size and the localization of the gland in relation to surgical landmarks were assessed with computed tomography (CT) in 130 dogs, and with magnetic resonance imaging (MRI) in the remaining 20 dogs. CT was performed in anesthetized dogs with a 3rd generation CT scanner (Tomoscan CX/S, Philips NV, Eindhoven, The Netherlands), using a protocol described previously.[19, 36] The height and width of the pituitary were measured on transverse images (Figure 1A). The length of the pituitary was estimated from the number of images containing a section of the gland.

MRI was performed in anesthetized dogs with a 0.2-Tesla open magnet (Magnetom Open Viva, Siemens AG, Germany) using a multipurpose coil. Contiguous 1-mm-thick transverse slices of the pituitary gland were obtained using a 3-dimensional flash (T1-weighted gradient echo) sequence both before and after the intravenous administration of 0.3 ml of contrast medium per kg of body weight (Dotarem, Guerbet Nederland BV, Gorinchem, The Netherlands, containing 279.32 mg gadoterate/ml as meglumine salt). The height and width of the pituitary were measured on transverse images and the length was measured on sagittal reconstructions of the transverse images (Figure 1B).

The maximum pituitary size was defined as the maximum value of pituitary height, width, or length. Enlarged pituitaries were distinguished from nonenlarged pituitaries by the ratio between the height of the pituitary gland and the area of the brain (P/B ratio), as

described previously.[16] 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.

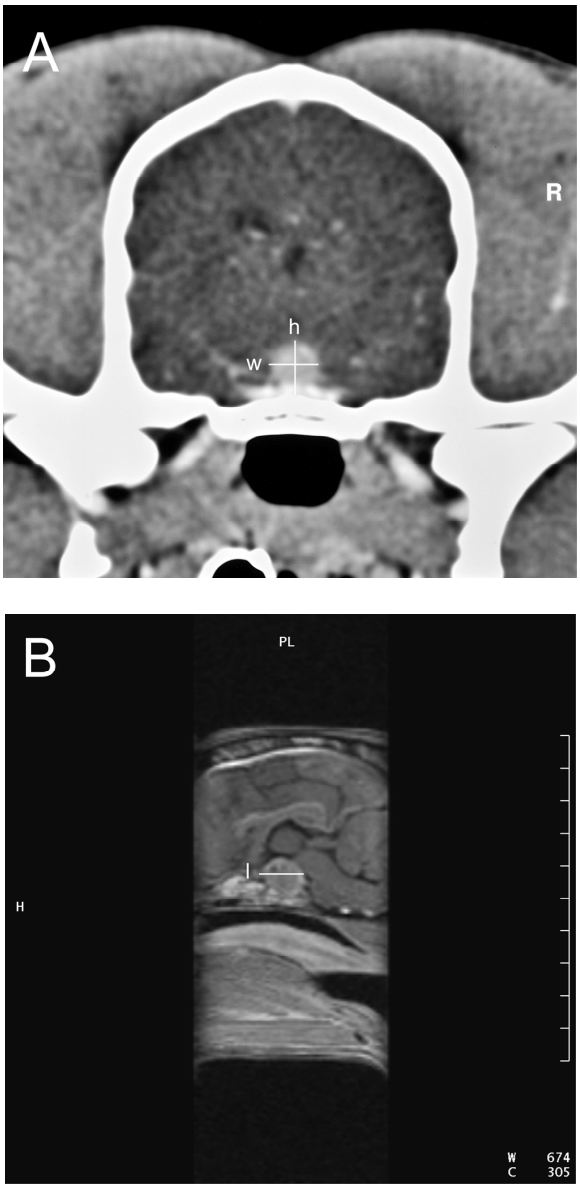

Figure 1. Measurements of pituitary dimensions (height, width, and length) in contrast-enhanced computed tomography (CT) (A) and magnetic resonance (MR) (B) images. (A) Transverse CT image: measurement of the pituitary height (h) and width (w) on transverse images. (B) Sagittal MR image: measurement of the pituitary length (l).

Surgical procedure and medication

Transsphenoidal hypophysectomy, and peri- and postoperative monitoring and medication were performed according to the protocol described earlier.[21,22] Usually, the dogs resumed drinking on the day of hypophysectomy, and were discharged from the hospital within 3 days after surgery. Hormone substitution consisted of life-long administration of cortisone acetate (Cortisoni acetas, Genfarma, Maarssen, The Netherlands) and thyroxine (L-thyroxine, Aesculaap, Boxtel, The Netherlands). Desmopressin (Minrin, Ferring, Hoofddorp, The Netherlands) was administered for 2 weeks routinely, and continued if polyuria due to central diabetes insipidus (CDI) persisted.[21] In case of postoperative decreased Schirmer tear test (STT) (Clement Clarke International Ltd, Harlow Essex, UK) values (≤5 mm wetting/min), dogs were treated according to a protocol as described earlier.[21]

Follow-up

After hypophysectomy, 136 dogs were reevaluated within 8 weeks, which included physical examination, routine blood chemistry, measurement of basal plasma T_4 concentration 10-12 hours after last thyroxine medication, and basal UCCR in duplicate at 24 hours after cortisone medication. After this 1st follow-up examination, UCCRs were measured at 6 months after surgery and thereafter once a year, unless a relapse was suspected in between. Urine samples were mailed to our laboratory, and follow-up reports were obtained from routine follow-up examinations in the hospital, and during telephone conversations with the owner, and/or the referring veterinarian. Postoperative mortality was defined as death within 4 weeks after surgery irrespective of the cause of death. Remission was defined as UCCR $<10 \times 10^{-6}$ and resolution of clinical signs of hyperadrenocorticism. Residual disease was defined as early postoperative (<8 weeks) UCCR $\geq 10 \times 10^{-6}$ and/or remnant pituitary tumor tissue on early postoperative (<2 months after surgery) CT or MRI scans. Recurrence was defined as UCCR $\geq 10 \times 10^{-6}$, return of clinical signs of hyperadrenocorticism or both after initial complete remission was achieved as defined above.

Statistical Analysis

Results are presented as median and ranges (UCCR, pituitary sizes). Survival and disease-free fractions were analyzed by the Kaplan-Meijer estimate procedure[10] as described previously.[21] Kaplan-Meijer curves for survival and disease-free fractions were plotted for dogs with a nonenlarged pituitary (P/B ≤0.31) and for dogs with enlarged pituitary tumors (P/B >0.31). In addition dogs with maximum pituitary dimension <10 mm were compared with dogs with maximum pituitary dimension ≥10 mm. Differences between Kaplan-Meier curves were tested for significance ($P < 0.05$) by the log rank test. The chi-square test was used to analyze the occurrence of keratoconjunctivitis sicca (KCS) and DI in relation to pituitary size.

Results

CT and MRI - preoperative scans

The pituitary glands, as measured on contrast-enhanced CT and MRI images, ranged in height (n=150) from 2.1 to 15 mm (median, 5.1 mm), in width (n=148) from 3.3 to 17 mm (median, 6.1 mm), and in length (n=143) from 2 to 18 mm (median, 5.0 mm). Pituitary

glands of 74 dogs were not enlarged, the P/B ratios ranged from 0.15 to 0.31 (median, 0.24). The pituitary glands of 76 dogs were enlarged with P/B ratios ranging from 0.32 to 0.76 (median, 0.43). The maximum dimension of the pituitary gland, ranged from 3.3 to 18.0 mm (median, 6.9 mm). The maximum diameter was <10 mm in 130 dogs ≥10 mm in 20 dogs.

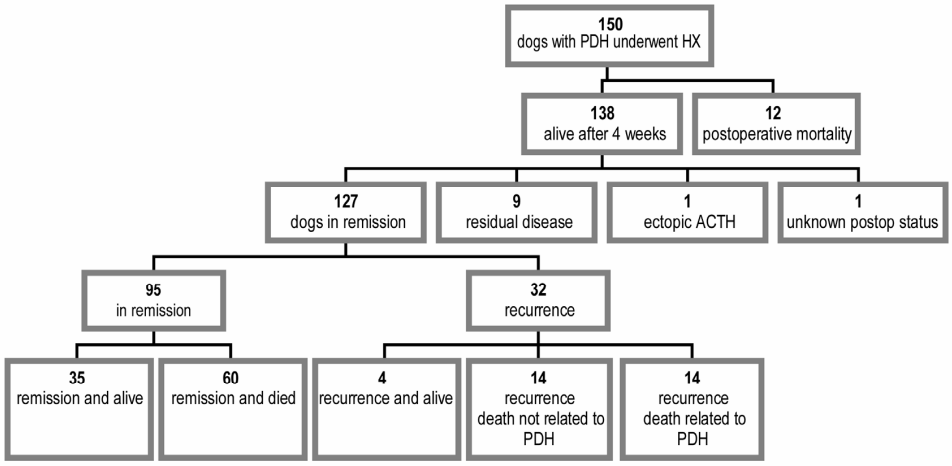

Figure 2. Organigram of 150 dogs with pituitary-dependent hyperadrenocorticism (PDH) that underwent transsphenoidal hypophysectomy (HX) in the period 1993-2003.

Postoperative mortality

Twelve dogs died within 4 weeks after surgery. Two dogs (dogs 3 and 27) with an enlarged pituitary (P/B ratios, 0.48 and 0.53, respectively) developed an arterial hemorrhage from the arterial cerebral circle during exploration of the fossa for pituitary remnants. Two dogs (dogs 115 and 132) died within 6 hours after surgery; postmortem examination in 1 dog (dog 132) revealed thromboendocarditis of the right atrium, concentric myocardial hypertrophy of the left ventricle and lung edema due to circulatory failure. Four dogs (dogs 8, 21, 105, 141), in which surgery was uneventful, died 1 day after surgery: 2 of these dogs (dogs 8, 105) became dyspneic, 1 dog (dog 21) with glucocorticoid-associated myotonia died of unknown cause, and 1 dog (dog 141) had hypernatremia due to insufficient oral fluid intake. This dog had been released prematurely to the surgical ward and died shortly after return to the intensive care unit. One dog (dog 148) died 5 days after surgery due to accidental IV injection of oral potassium solution. Two dogs had a prolonged stay in the ICU for 2 weeks because of severe hypernatremia (dog 5), and diabetic ketoacidosis (dog 65) and were eventually euthanized. One dog (dog 14) developed severe bronchopneumonia, had a prolonged stay in the hospital (10 days) and died at home 4 weeks after surgery.

Long-term survival and disease-free fraction

One-hundred-thirty-eight dogs were alive 4 weeks after surgery. Hyperadrenocorticism went into remission in 127 dogs (Figure 2). In 9 dogs, there was residual disease (Figure 2) based on early high UCCR ($\geq 10 \times 10^{-6}$) and/or remnant pituitary tissue on postoperative pituitary

imaging within 8 weeks after surgery. Five of the 9 dogs with residual disease were euthanatized or died within 5 months after surgery for reasons associated with hyperadrenocorticism, 3 dogs were treated with o,p'DDD (Lysodren; Bristol-Meyers, Syracuse, NY) 3 to 6 months after surgery and, at the time of evaluation, survival times were 17, 26, and 32 months. In 1 dog with residual disease bilateral adrenalectomy was performed. The dog survived 34 months until it developed seizures and was euthanatized. In 1 other dog, clinical signs worsened and UCCR further increased after surgery (Figure 2). In this dog, complete hypophysectomy was confirmed with postoperative CT (empty sella) and at histopathological examination of the complete pituitary, no pituitary adenoma was found. Based on persistent high plasma ACTH concentrations it was suspected that this dog had an extrapituitary source of ACTH-secretion. Following total body CT scanning and laparotomy for abdominal masses, a metastasized neuroendocrine pancreatic tumor was found.[11] One dog died at home 7 weeks after surgery because of renal failure; no postoperative UCCR was available (Figure 2).

In 124 dogs in which hyperadrenocorticism went into remission, basal UCCR values within 8 weeks after hypophysectomy were $<1 \times 10^{-6}$ in 50 dogs, ≥ 1 - $<5 \times 10^{-6}$ in 55 dogs, and ≥ 5 - $<10 \times 10^{-6}$ in 19 dogs. In 3 dogs, there was late remission, signs of hyperadrenocorticism resolved, and low basal UCCR values (5.1, 4.5, 3.9 x 10^{-6}) were achieved or available later than 8 weeks, at, respectively, 3, 6, and 36 months after hypophysectomy.

The long-term follow-up results are presented by curves of estimated survival and disease-free fraction (Figures 3A, 4A). The 1-year estimated survival rate was 83.5% (95% confidence interval [CI], 76-89%). The 2-year estimated survival rate was 76.1% (95% CI, 67-83%). The 3-year estimated survival rate was 71.5% (95% CI, 62-79%). The 4-year estimated survival rate was 67.8% (95% CI, 55-77%)(Figure 3). The 1-year estimated relapse-free fraction was 87.9% (95% CI, 80-93%). The 2-year estimated relapse-free fraction was 74.9% (95% CI, 64-83%). The 3-year estimated relapse-free fraction was 66.3% (95% CI, 54-76%). The 4-year estimated relapse-free fraction was 58.5% (95% CI, 45-70%)-(Figure 4).

In 95 of 127 dogs (75%), hyperadrenocorticism remained in remission (Figure 2). Over time 60 of these 95 dogs died (9 dogs) or were euthanatized (51 dogs) because of non-Cushing-related causes after a median interval of 28 months after hypophysectomy (range, 2-87 months). The causes of death were: old age (13 dogs), carcinoma (11 dogs), heart failure (9 dogs), preexisting primary epilepsy (5 dogs), gastrointestinal problems (5 dogs), preexisting diabetes mellitus (4 dogs), passive urinary incontinence (4 dogs), anterior or posterior paralysis (3 dogs), pancreatitis (1 dog), pre-existing copper-associated hepatitis (1 dog), chronic renal failure (1 dog), lung failure (1 dog), skin problems (1 dog) and behaviour problems (1 dog). In the 35 dogs alive at the time of evaluation, the period of remission ranged from 2 to 89 months (median, 15 months). In these 35 dogs, median P/B ratio was 0.29 (range, 0.15-0.71); in 20 dogs, the pituitaries were nonenlarged (P/B ratio ≤ 0.31); and in 15 dogs, the pituitaries were enlarged (P/B ratio >0.31). The median value of the last available UCCR was 0.6 x 10^{6}, which was not significantly different from the median value 0.8 x 10^{-6} within 2 months after hypophysectomy.

In 32 of 127 dogs (25%) (Figure 2), signs of hyperadrenocorticism recurred with high UCCR ($\geq 10 \times 10^{-6}$) at 6 weeks to 56 months after surgery (median, 18.3 months). In the 32 dogs in which hyperadrenocorticism recurred, the median basal UCCR at time of initial

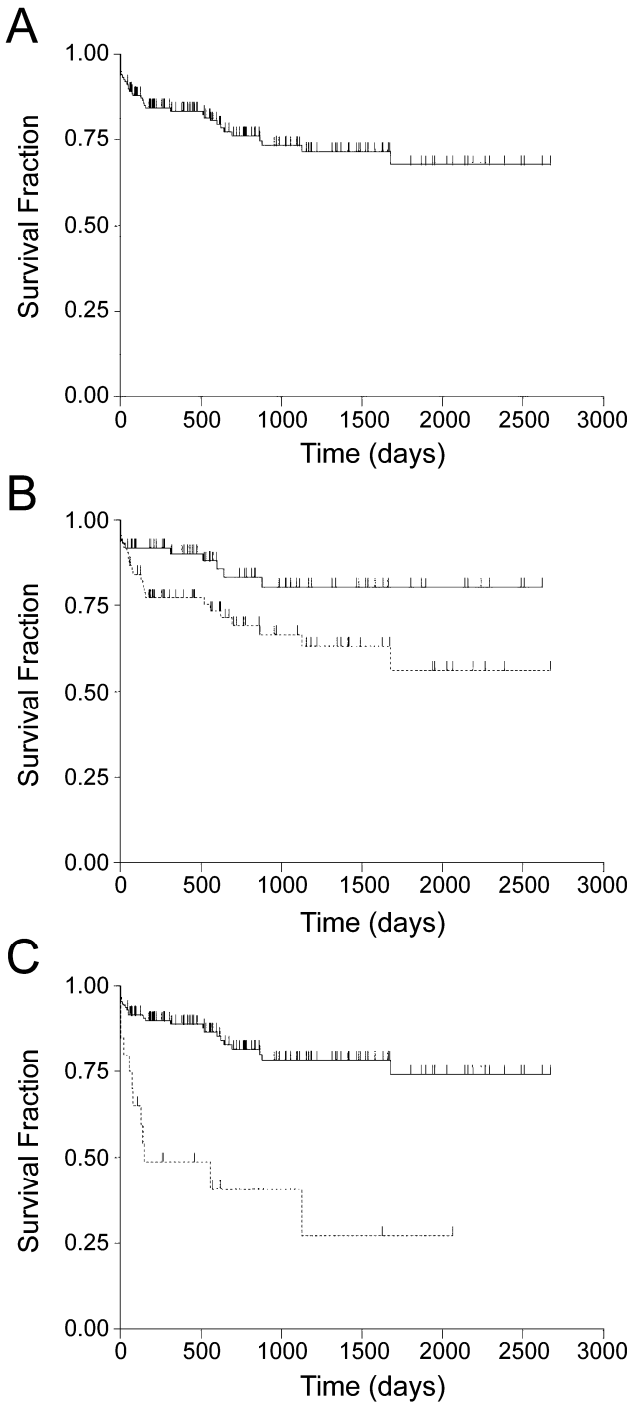

Figure 3. (A) Survival curve after transsphenoidal hypophysectomy for 150 dogs with pituitary-dependent hyperadrenocorticism. Censored cases (i.e., dogs that died from unrelated causes or were still alive at the time of follow-up) are represented by vertical bars. (B) Survival curves for dogs with pituitary/brain ratio (P/B) ≤0.31 (—) and dogs with P/B >0.31 (---) (Log rank test, P=0.023). (C) Comparison of survival curves for dogs with pituitary diameter <10 mm (—) and dogs with pituitary diameter ≥10 mm (---) (Log-rank test, P <0.001).

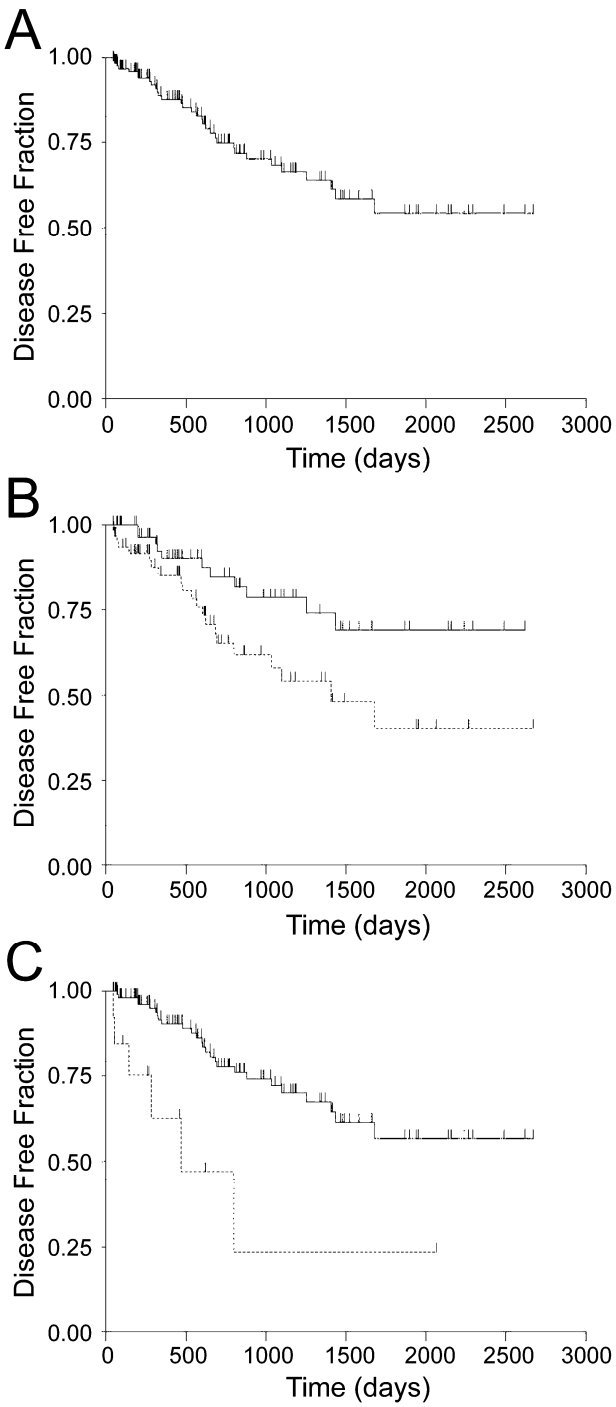

Figure 4. (A) Disease-free fraction curve after transsphenoidal hypophysectomy for 127 dogs with pituitary-dependent hyperadrenocorticism. Censored cases (i.e., dogs that died from unrelated causes or were still alive at the time of follow-up) are represented by vertical bars. (B) Comparison of disease-free fraction curves for dogs with pituitary/brain ratio (P/B) ≤0.31 (—) and dogs with P/B >0.31 (---) (log rank test, P=0.020). (C) Comparison of disease-free fraction curves for dogs with pituitary diameter <10 mm (—) and dogs with pituitary diameter ≥10 mm (---) (log rank test, P <0.001).

remission was 3.7 x 10^{-6}. The UCCR were <1 x 10^{-6} in 6 dogs, ≥1 and <5 x 10^{-6} in 14 dogs, and ≥5 and <10 x 10^{-6} in 12 dogs. The median P/B ratio before surgery in these 32 dogs was 0.40 (range, 0.19-0.76), in 11 dogs the pituitaries were nonenlarged (P/B ratio ≤ 0.31); and in 21 dogs the pituitaries were enlarged (P/B ratio >0.31). One dog (dog 124) developed a recurrence 16 months after hypophysectomy and went in remission spontaneously two months later. Nevertheless the dog was categorized in the recurrence group. Fifteen of the 32 dogs with recurrence were subsequently treated with o,p'DDD , and 5 dogs received no further therapy at the owners request. At the time of evaluation, 14 of the 32 dogs were euthanatized or died because of recurrent signs of hyperadrenocorticism, 14 died of unrelated causes and 4 dogs were still alive.

Survival and disease-free fractions of dogs with enlarged pituitaries (P/B >0.31) were significantly (log rank test, P=0.023 and P=0.020, respectively) lower than in dogs with nonenlarged pituitaries (Figures 3B, 4B). Survival and disease-free fractions of dogs with pituitary dimension ≥10mm were significantly (P <0.001) lower than those of the dogs with pituitary dimension <10 mm (Figures 3C, 4C).

Keratoconjunctivitis sicca (KCS)

In 58 eyes in 47 dogs of the total of 150 dogs (31%), there was no (STT = 0 mm in 26 eyes) or decreased (STT values >0 and ≤ 5 mm wetting/min in 32 eyes) tear production on the 1st postoperative day. The dogs had blepharospasm and conjunctivitis and were treated according to a KCS. KCS developed significantly more frequently (chi-square test, P <0.001) in the left eye (38 eyes) than in the right eye (20 eyes). STT values became normal in 29 left eyes and in 18 right eyes. In 11 eyes in 10 dogs (6.7%), tear production remained low until death. Ophthalmologic treatment was needed for 3-547 days in recovered left eyes (median 70 days) and for 3-717 days in recovered right eyes (median 58 days). In the 1st series (dog 1 through 75), 32 eyes in 26 dogs (35%), and in the 2nd series (dogs 76-150), 27 eyes in 21 dogs (28%) developed KCS. There was no relation between the frequency of KCS and pituitary size.

Central Diabetes Insipidus (CDI)

In 67 of 127 dogs (53%) in which there was remission of hyperadrenocorticism, prolonged (more than 2 weeks) treatment with desmopressin was needed to control polyuria. In 28 of 127 dogs (22%), CDI was present until death or until the latest available follow-up date and treatment with 1 or 2 drops desmopressin was required. In the other 39 of 127 dogs (31%), desmopressin was discontinued after 28-1,329 days (median, 133 days). Of the dogs in remission, CDI occurred in 29 dogs (47%) of the 1st series (*n*=62), and in 38 dogs (58%) of the 2nd series (*n*=65), and CDI occurred significantly more frequently (chi-square test, P=0.04) in dogs with a P/B ratio >0.31 (39 of 63 dogs =62%) than in dogs with P/B ratio ≤0.31 (28 of 64 dogs = 44%).

Discussion

Since our report on the results of transsphenoidal hypophysectomy in 52 dogs with PDH,[21] the number of operated dogs has increased to 150 dogs. The extended follow-up on these dogs confirms that transsphenoidal surgery is an effective treatment of PDH in dogs. The

results were obtained as a joint effort of the disciplines endocrinology, diagnostic imaging, and neurosurgery, with all hypophysectomies performed by 1 neurosurgeon (BPM). Also in human medicine, transsphenoidal surgery is the treatment of choice for Cushing's disease,[23] and pituitary surgeries at a center should best be performed by 1 surgeon to ascertain enough volume and to maintain the surgical skills.[1,26]

In the present study there were 12 deaths within the 1st month after surgery and 7 of these were in the 1st series of 75 dogs and 5 in the 2nd series of 75 dogs. All 5 dogs in the 2nd series died in the ICU, and 2 deaths were related to errors in the ICU. The reduction in surgical mortality (death within 4 weeks after surgery irrespective of cause of death) in the previous study on 52 dogs was attributed to the initial learning curve for the surgical procedure and to improvements in postoperative care.[21] In the present study, the post-operative mortality in the 2nd 75 dogs is restricted to the immediate postoperative period in the ICU. Further reduction in postoperative mortality may be expected from a more stringent ICU protocol for hypophysectomies. The results in the 2nd 75 dogs approach the mortality rate (1-4%) reported for transsphenoidal surgery for Cushing´s disease in humans.[4,17,26]

Depending on the definition of remission criteria, the remission rate varies between 42-93% after pituitary surgery in humans with Cushing's disease.[26,32] In humans, the corticotroph adenomas are usually microadenomas and may be so small that they may be difficult to localize with pituitary imaging or during pituitary surgery.[32] Recurrence is most likely to be caused by regrowth of adenoma cells left in situ.[21] After transsphenoidal hypophysectomy in healthy dogs, the pituitary fossa was usually found to contain micro-scopic nests of pituitary cells.[22]

In the present study, remission was achieved in 127 dogs of 150 dogs (85%). Remission was defined as UCCR $<10 \times 10^{-6}$ and resolution of clinical signs of hyperadrenocorticism. Recurrence rate was 25% (32 of 127 dogs). In two other studies, 11 and 78 dogs with PDH were treated with trilostane, a competitive inhibitor of adrenal 3β-hydroxysteroid dehydro-genase. Remission of polyuria/polydipsia occurred in 100% and 70% of the dogs, res-pectively; skin abnormalities improved in 82% and 62%, respectively.[24,30] In another study with 30 dogs, trilostane treatment reduced plasma cortisol concentrations, and clinical signs improved.[6] However, follow-up periods for trilostane have not been long enough to allow comparison with the results of hypophysectomy. The remission and recurrence rates in the present study compare favorably with those of 129 dogs with PDH treated at the same institution with o,p'DDD for nonselective destruction of the adrenal cortex.[7] Using similar criteria, remission in that study occurred in 111 dogs (86%) of which 43 (39%) had a relapse (UCCR $>10 \times 10^{-6}$).

If all procedure-related mortalities (12 dogs), incomplete hypophysectomies (9 dogs), and recurrences after remission (32 dogs) are considered to be treatment failures, the overall success rate in this study was 65% (97 of 150 dogs). These results compare favorably with those of 129 dogs treated in the same institution with o,p'DDD.[7] The overall success rate in the o,p'DDD study was 61% (68 of 111 dogs), the estimated 1-, 2-, and 3-year survival fraction were 80%, 69%, and 61%, respectively.[7] In the present surgical study the 1-, 2-, and 3-year survival fraction were 84%, 76%, and 72 %, respectively. The estimated 1-, 2-, and 3-year disease-free fraction in the o,p'DDD study were 77%, 53%, and 44%, respectively. In the present surgical study the 1-, 2-, and 3-year disease-free fraction were 88%, 75%, and 66%, respectively. Thus, with longer follow-up time hypophysectomy leads to better results than o,p'DDD treatment. However, there may have been a bias for selection of dogs for

surgery (smaller tumors) or for o,p'DDD treatment (larger tumors). Still, in this study 76 dogs (50%) had enlarged pituitaries with increased P/B ratio.

Comparison with the results of studies performed in other institutions is more difficult. In a study of o,p'DDD treatment in 54 dogs, the 1-, 2-, and 3-year survival fractions were 80%, 59%, and 45%, respectively.[9] This study included dogs with adrenocortical tumors. In another study including 200 dogs with PDH, 72% of the dogs were alive after 1 year, 47% after 2 years and 30% after 3 years of chronic (non-selective) treatment with o,p'DDD.[15] In a study of 78 dogs treated with trilostane, the median survival time of the 26 dogs which died was 549 days.[24] The experience with radiotherapy as a treatment for dogs with PDH is limited. In a study using cobalt 60 radiotherapy in 6 dogs with PDH, the clinical signs resolved in 3 dogs but recurred in 2 dogs within 10 months. There was a substantial decrease in tumor size 1 year after radiotherapy.[12] Megavoltage irradiation in dogs has also been shown to reduce tumor size.[35]

There is a significant influence of pituitary tumor size, reflected in the maximum pituitary dimension and P/B ratio, on survival and disease-free fraction after hypophysectomy in dogs with PDH. The larger the pituitary tumor, at time of surgery, the shorter survival and disease-free fraction after surgery. In humans with pituitary adenomas the incidence of dural invasion increases with pituitary size, and dural invasion is correlated to a decreased survival rate.[20] In other studies on transsphenoidal surgery in humans with Cushing's disease, the remission rate of cases with suprasellar lesions has been found to be lower than cases of intrasellar lesions,[26,31,34] with a correlation of remission rate and size of the pituitary.[26] The influence of the size of microadenomas on remission rate has been studied by Shimon et al.[32] The patients were divided into three groups; no detectable tumor on MRI, tumor size 2-5 mm and tumor size 6-10 mm. All pituitary tumors were restricted to the sellar compartment which, in humans, measures 10 mm in diameter. There was no difference in remission rate between the groups.[32] These findings demonstrate the importance of early diagnosis of a corticotroph adenoma. The period of remission after surgery is expected to be the longest when the pituitary adenoma is still contained in the sellar compartment.

After complete hypophysectomy, there is a sudden cessation of arginine vasopressin (AVP) secretion by the neurohypohysis. Usually, after a few days, AVP released by the hypothalamic paraventricular and supraoptic nuclei reaches the systemic circulation sufficiently to restore antidiuresis.[21] The transient CDI normalizes 5 days after hypophysectomy in healthy dogs, but may persist up to 2 weeks in dogs with PDH,[12, 13] and severe hypernatremia after hypophysectomy in dogs has been reported.[18] After transsphenoidal pituitary surgery in humans, transient CDI lasting 1-3 days occurs in 38% of the patients.[17,26] Desmopressin, a synthetic AVP analog, is administrated to substitute for the postoperative impairment of AVP release. The prophylactic efficacy of desmopressin after hypophysectomy in healthy dogs, has been studied by Hara et al.[13] Administration of 4 µg desmopressin acetate in the conjunctival sac twice daily effectively prevented hypernatremia that otherwise occurred 24 hours after surgery. However, mild immediate postoperative hypernatremia was frequently observed after hypophysectomy in dogs with PDH that were routinely treated with desmopressin postoperatively for a period of 2 weeks.[21] This may be explained by the vasopressin resistance in dogs with hyperadrenocorticism.[3] Therefore, to prevent hypernatremia, low sodium fluids (0.45% sodium chloride + 2.5% glucose) are best started before and continued during and after surgery.[21] Thirty-nine dogs in the present study developed chronic CDI, which required desmopressin administration for relatively long

periods until it could be discontinued. The damage to the axons of pituitary stalk may be such that neuronal degeneration ensues, leading to permanent CDI.[22] In our study, permanent CDI developed in 28 of 127 dogs, which is more frequent than in humans after transsphenoidal surgery.[23] The explanation for this is that, in humans with Cushing's disease, selective adenomectomy is performed, whereas in the present study total hypophysectomy was performed, which includes removal of the neurointermediate lobe. The frequency of permanent CDI in the present study is also higher than that in the previous study (10%, 5 of 52 dogs).[21] Pituitary tumor extension usually occurs in dorsal direction and prolonged mass effect by the tumor on the hypothalamic nuclei may result in (irreversible) damage to these nuclei. Indeed, the incidence of CDI was significantly higher in the group of dogs with an enlarged pituitary than that in the group of dogs with a nonenlarged pituitary. Also, efforts to completely remove dorsally located tumor tissue in cases with large pituitary tumors may lead to damage of the median eminence, leading to more frequent CDI than in dogs with small pituitary tumors.

KCS is a severe complication after transsphenoidal hypophysectomy in dogs if left untreated. Decreased tear production was detected in 47 of 150 dogs in the present study, which is similar to what was found in the previous study, 18 of 52 dogs.[21] With early detection, KCS develops less severely. Routine postoperative check of the STT values and immediate ophthalmologic treatment prevent the development of lesions in the cornea. Complete recovery occurred in 47 eyes after intensive treatment, but in 11 eyes of 10 dogs, the tear production remained low until death. There was no correlation between pituitary size and development of KCS.

KCS after hypophysectomy has been ascribed to direct (traumatic) or indirect (ischemic) neuropraxia of the major petrosal nerves, resulting in a secretomotoric deficit in the lacrimal glands.[22] Decreased tear production occurred more frequent in the left than in the right eye, a finding consistent with earlier studies.[21,22] It may be that a right-handed surgeon deviates the burr slot slightly to the left side of the dog, leading to more unilateral petrosal nerve damage. Another explanation for occurrence of KCS is ischemic damage to the pterygopalatine ganglion from longstanding pressure of the mandibular coronoid process in the retrobulbar area due to the open mouth approach. This theory is supported by the fact that the degree of lower jaw retraction has successively been reduced with time, and the incidence of KCS in the latest dogs seems to decrease (data not shown). This theory, however, cannot fully explain the predominance of decreased tear production in left eyes.

The lacrimal gland has been shown to be dependent on pituitary hormones. Reduced tear production has been reported in association with endocrine diseases such as hyperadrenocorticism (Peirce VE, Williams DL. Reduced tear production in 50 dogs with endocrine disease [abstract]. In Proceedings of British Small Animal Veterinary Association Congress, Birmingham, England, 2004:517), which may also have affected the occurrence of KCS after hypophysectomy. Although the pathogenesis of the decreased tear production after transsphenoidal hypophysectomy in dogs remains uncertain, it may be concluded that the STT should be performed on the 1st day after hypophysectomy. Early detection and treatment of a reduction in tear production prevents the development of KCS. In most cases tear production recovers with time.

It is concluded that hypophysectomy is an effective long-term treatment for PDH in dogs directed at elimination of the pituitary origin of the disease. With increasing pituitary dimensions, the survival and disease-free fractions after hypophysectomy decrease, and the inci-

dence of DI increases. Therefore, early diagnosis of a corticotroph adenoma is important and transsphenoidal hypophysectomy is expected to have the best outcome when installed as primary treatment for dogs with nonenlarged or moderately enlarged pituitaries.

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CHAPTER 4

Prognostic factors for outcome after transsphenoidal hypophysectomy in dogs with pituitary-dependent hyperadrenocorticism

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Abstract

The aim of this study was to determine prognostic factors for outcome after transsphenoidal hypophysectomy in dogs with pituitary-dependent hyperadrenocorticism (PDH).

Transsphenoidal hypophysectomy was performed in 181 dogs with PDH by one veterinary neurosurgeon during a 12-year period. Survival analyses were performed with the Kaplan-Meijer estimate procedure. Prognostic factors (including patient, hormone and imaging data) were analyzed by univariate Cox's proportional-hazard analysis followed by stepwise multivariate analysis. The log-rank test was used to assess disease-free fractions in three groups categorized by the early postoperative urinary corticoid-to-creatinine ratio (UCCR).

Multivariate analysis revealed that old age, large pituitary size, and high plasma adrenocorticotropic hormone (ACTH) concentrations before surgery were associated with an increased relative risk for PDH-related mortality. Large pituitary size, relative thick sphenoid bone, high UCCR, and high plasma α-melanocyte-stimulating hormone (α-MSH) concentration before surgery were associated with an increased risk of recurrence in dogs that went into remission after hypophysectomy. Disease-free fractions were significantly higher in dogs with postoperative UCCR in the low normal range ($< 5 \times 10^{-6}$) than in dogs with postoperative UCCR in the upper normal range ($5\text{-}10 \times 10^{-6}$).

The results of this study indicate that pituitary size, thickness of the sphenoid bone, plasma α-MSH concentration, and urinary cortisol excretion before surgery are predictors for long-term remission after transsphenoidal hypophysectomy for the treatment of PDH in the dog. UCCR measured at 6 to 10 weeks after surgery can be used as guidance for predicting the risk of recurrence.

Pituitary-dependent hyperadrenocorticism (PDH) or Cushing's disease is a common endocrine disorder in dogs accounting for 85% of the cases of spontaneous hypercortisolism,[50] which is reported as an animal model for Cushing's disease in humans.[29]

In dogs, hypophysectomy is performed and this approach has proven to be effective.[24,34] Transsphenoidal selective adenomectomy is the treatment of choice for Cushing's disease in humans.[3,7,31] The short-term surgical outcome after pituitary surgery is better for surgeons and hospitals with a high case load.[3] Humans with Cushing's disease have a higher risk of complications after pituitary surgery compared to patients with other pituitary tumors.[3]

Although remission rates are initially high (70-90%, reviewed by others),[2,41,42,49] recurrences are common in both species especially in the long term.[2,7,12,13,24,25] Preoperative variables associated with decreased surgical outcome in humans are increased pituitary size,[6,8,11,12,18,22,32,41,49,60] and dural invasion of the pituitary adenoma.[6,11,14,18,35,41] Male gender, large pituitary tumor size, and extrasellar extension are risk factors for residual disease after surgery.[6,22,32] In both species, disease-free fractions are lower in cases with macroadenomas than in cases with microadenomas.[6,24,60] Furthermore, age, inability to identify pituitary tumor on preoperative computed tomography (CT) or magnetic resonance imaging (MRI), severity of clinical signs, depression and high pre-treatment urinary cortisol levels are reported to be risk factors for recurrence.[7,57]

Postoperative cortisol measurements are used for defining surgical failure and remission in human patients.[2,12,17,45,46,49,54,56,61,66] However, there is no consensus with regard to the interpretation of postoperative cortisol values.[15,44] Low postoperative serum cortisol concentration on the day after surgery before glucocorticoid therapy is initiated[54] or low cortisol concentrations at 3 months after pituitary surgery when glucocorticoids are routinely used[45] have been reported to be associated with a low risk for long-term recurrence. However, for a subgroup of patients, postoperative serum cortisol concentrations do not accurately predict surgical outcome.[2,45,49,66]

Dogs with PDH and enlarged pituitary glands have significantly lower survival and disease-free fractions after transsphenoidal hypophysectomy than dogs with normal sized pituitary glands.[24] Also, the presence of adrenocorticotropic hormone (ACTH) pulses after hypophysectomy is a risk factor for the recurrence of hyperadrenocorticism.[23] The aim of the present study was to report the outcome of hypophysectomy at one center by one veterinary neurosurgeon for treatment of 181 dogs with PDH with a follow-up time up to 12 years. Prognostic factors were analyzed for early post-operative mortality and recurrences of canine PDH after transsphenoidal hypophysectomy.

Materials and Methods

Animals

One-hundred-and-eighty-one dogs with PDH, referred to the Department of Clinical Sciences of Companion Animals, Utrecht University, over a 12-year (1993–2005) period, underwent transsphenoidal hypophysectomy as primary treatment for PDH. Purebred dogs of 57 different breeds and crossbred dogs were represented. The most common breeds were Dachshund (n=16), Minature Poodle (n=13), Maltese (n=8) and Yorkshire Terrier (n=8), which together comprised 25% of the dogs. Eighty dogs were male (25 castrated) and 101 were female (62 spayed). The age at the time of surgery ranged from 3 to 14 years

(median, 9 years). The body weights ranged from 4 to 61 kg (median, 15 kg) with 88 dogs in the group 0 to ≤ 15 kg, 60 dogs in the category > 15 to ≤ 30 kg and 33 dogs weighed > 30 kg. In addition, the dogs were divided into three groups according to the shape of the skull: brachycephalic (*n*=8), mesaticephalic (*n*=125) and dolicocephalic (*n*=10). The 38 crossbred dogs were not categorized by skull shape.

Most dogs had the classical features of canine hyperadrenocorticism such as polyuria, polyphagia, truncal obesity, pot-belly appearance, muscle atrophy, and skin changes such as skin atrophy, alopecia, and calcinocis cutis (Figure 1A).[50] A few dogs also showed neurological signs due to tumor mass effect.[50]

Diagnosis

The diagnosis of hyperadrenocorticism was based upon urinary corticoid-to-creatinine ratio (UCCR) in two consecutive morning urine samples combined with a high-dose dexamethasone test, as described earlier.[53,59] After collection of the second urine sample, three oral doses of 0.1 mg dexamethasone per kg body weight were administered at 8-h intervals and the next morning a third urine sample was collected. When the UCCR in the third sample was less than 50% of the mean in the first 2 samples, the dog was categorized as being responsive to dexamethasone suppression and PDH was diagnosed.[20] In cases with less than 50% suppression dexamethasone-resistent PDH was confirmed by measurements of plasma ACTH concentrations and further supported by visualization of the adrenals by ultrasonography and pituitary imaging.[10,52,63,64]

Imaging

Pituitary imaging was performed in anesthetized dogs with a third generation CT scanner (Tomoscan CX/S, Philips NV, Eindhoven, The Netherlands) (155 dogs) (Figure 2) or with a 0.2 T open field MRI scanner (Magnetom Open Viva, Siemens AG, München, Germany) (26 dogs) using protocols described previously.[24,62] The height and width of the pituitary gland were measured on transverse images. The length of the pituitary gland was estimated from the number of images containing a section of the gland (CT) or on sagittal reconstructions of the transverse images (MRI). The ratio between height of the pituitary gland and the area of the brain (P/B ratio) was calculated to correct for the large differences in dog size.[30] Pituitaries with a P/B ratio > 0.31×10^{-2} mm^{-1} were enlarged and those with a P/B ratio ≤ 0.31×10^{-2} mm^{-1} nonenlarged.[30] The pituitary height-to-width, height-to-length, and width-to-length ratios were determined to estimate pituitary shape. Pituitary volume was calculated with an ellipsoid approximation model ($\pi/6$ x the product of the length, width and height).[4] The thickness of the sphenoid bone was measured on the transverse image on which pituitary height was measured.

Surgery

Transsphenoidal hypophysectomy was performed according to a microsurgical technique described previously.[39] Postoperative hormone substitution therapy consisted of cortisone acetate and thyroxine.[38] Desmopressin was administered for 2 weeks and continued if polyuria due to central diabetes insipidus persisted.[24,38,39] Re-examination after 8 weeks included physical examination, routine blood chemistry, measurements of basal plasma thyroxine concentration at 10 to 12 h after L-thyroxine medication, and basal UCCR urine samples

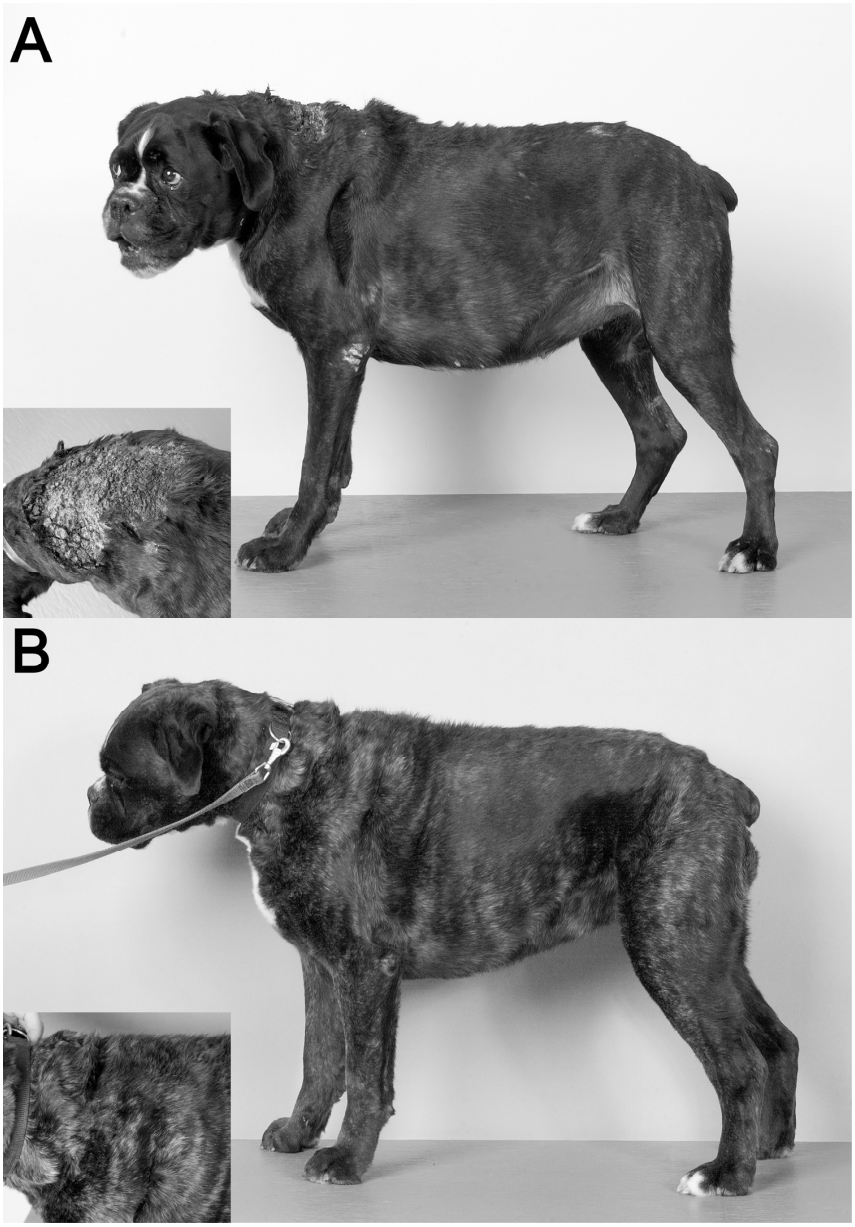

Figure 1. (A) A 5-year-old female Boxer dog with features of (pituitary-dependent) hyperadrenocortisim: abdominal enlargement, atrophy of the thigh muscles, some hair loss in the groins, and severe secondary crusty dermatitis along the dorsum with calcinosis cutis (see insert from shoulder). The dog had a pronounced polydipsia, polyuria and a ravenous appetite. (B) 3 months after hypophysectomy with less sagging abdomen, full recovery of the hair coat, and regain of muscle mass (see insert from shoulder) (See Color section).

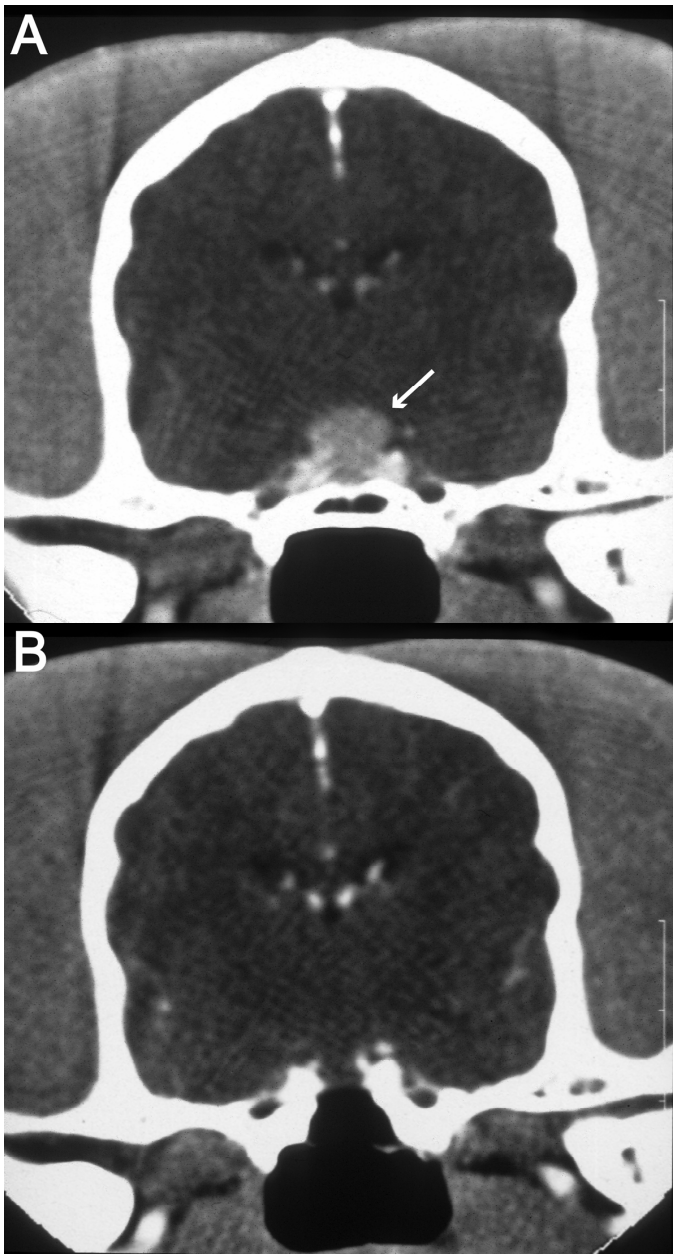

Figure 2. (A) A typical computed tomography image of the skull of 13-year-old female crossbred dog with hyperadrenocorticism due to a large pituitary adenoma (arrow). (B) The same dog 8 weeks after hypophysectomy.

collected at home at 24 h after cortisone medication. UCCRs were measured again 6 months after surgery and thereafter once a year. In case of suspicion of recurrence, UCCR was determined earlier.

Postoperative mortality was defined as death within 4 weeks after surgery irrespective of the cause of death. Residual disease was defined as early postoperative (< 2 months after surgery) UCCR $\geq 10 \times 10^{-6}$ and no resolution of clinical signs and/or remnant pituitary tumor tissue on early postoperative CT or MRI scans. Remission was defined as UCCR $< 10 \times 10^{-6}$ and resolution of clinical signs of hyperadrenocorticism. Recurrence was defined as UCCR $\geq 10 \times 10^{-6}$ and return of clinical signs of hyperadrenocorticism after initial complete remission (Figure 1B).

Hormone Determinations

Plasma ACTH concentration was measured by two different methods during the course of the study. In 59 dogs a radioimmunoassay (RIA) without extraction was used, according to the procedure validated for the dog and described previously.[1,10,40] This antiserum also cross reacted with ACTH precursors. The tracer was purchased from International CIS (St Quentin-Yvelines, France), and the standard was obtained from the NIH (Bethesda, MD, USA). The intra-assay coefficient of variation (CV) was 8%, the inter-assay CV was 12%, and the sensitivity was 2.2 pmol/l. The cross-reactivity with α-melanocyte-stimulating hormone (α-MSH) was < 0.1%.[10]

In 112 dogs plasma ACTH concentrations were measured using a commercially available two-site immunoradiometric assay (IRMA) (Nichols Institute, Wijchen, The Netherlands). The antiserum is highly specific for ACTH (1-39). The intra-assay CV was 3.2%, the inter-assay CV was 7.8%, and the sensitivity was 0.22 pmol/l. There was no cross-reactivity between the antiserum and α-MSH or ACTH precursors.[10,47]

Plasma cortisol concentrations were measured with two comparable methods. In 72 dogs a RIA was used, using cortisol antiserum as described previously.[51] The intra-assay CV was 5%, the inter-assay CV was 10% and the sensistivity was 1 nmol/l. In 93 dogs plasma cortisol concentrations were measured by a solid phase ^{125}I RIA (Coat-A-Count® Cortisol, Diagnostic Products Corporation, Los Angeles, USA). The antiserum is highly specific for cortisol, with very low cross-reactivity to other compounds that are present in patient samples. The intra-assay CV was 4% the inter-assay CV was 4.5-6.3%, and the sensitivity was 5.5 nmol/l.

The urinary corticoid concentration was measured by RIA as described previously.[53] The intra-assay CV was 6%, the inter-assay CV 8%, and the sensitivity was 1 nmol/l. The urinary corticoid concentration was related to the urinary creatinine concentration (Jaffé kinetic method, initial rate reaction) and the UCCR was calculated.[53,59]

Plasma concentration of α-MSH was measured by RIA without extraction according to methods described previously.[51] The intra-assay CV was 10%, the inter-assay CV was 23%, and the sensitivity was 3 pmol/l. The antiserum had less than 0.1% cross-reactivity with ACTH (1-39) and 4% cross-reactivity with ACTH (1-24). Plasma α-MSH concentrations equal to or more than 36 pmol/l were considered increased.[10]

According to protocol previously described, plasma growth hormone (GH) concentration was measured by a homologous RIA[16] plasma prolactin (PRL) and luteinizing hormone (LH) concentration were measured by heterologous RIAs,[43,58] plasma thyroid stimulating hormone (TSH) concentration was measured by a homologous IRMA using a commercially available kit (Diagnostic Products Corp., Los Angeles, USA),[65] and plasma total thyroxine (T_4) con-

centration was measured by RIA, with essentially the same method as was described by Belshaw and Rijnberk.[5]

Statistical Analysis

Survival analyses were performed with EGRET (Cytel Inc., Cambridge, MA, USA) statistical packages. Survival and disease-free fractions were calculated according to the Kaplan-Meier estimate procedure.[19] The survival period was defined as the interval between the date of the surgery and the date on which the dog was last known to be alive or the date of its death due to any cause. The disease-free interval was calculated for the dogs in which remission of hyperadrenocorticism was obtained, and was defined as the interval between the date of surgery and the date on which the dog was last known to be free of signs of hyperadrenocorticism and to have UCCR $< 10 \times 10^{-6}$, or the date of recurrence of signs of hyperadrenocorticism and UCCR $\geq 10 \times 10^{-6}$. Dogs that had died from non-related causes and dogs that were still alive at the time of follow-up were counted as censored cases. Prognostic factors, expressed as hazard ratios, were first analyzed by univariate Cox's proportional-hazard analysis. The following variables were analyzed; age (n=181), gender including castration status (n=181), body weight (n=181), mean UCCR before surgery (n=180), UCCR after high-dose dexamethasone suppression (n=180), degree of dexamethasone suppression on UCCR (n=180), pituitary height (n=181), pituitary width (n=181), pituitary length (n=178), pituitary height-to-width ratio (n=181), pituitary height-to-length ratio (n=178), and pituitary width-to-length ratio (n=178), P/B ratio (n=180), pituitary volume (n=178), pituitary enlargement (n=180), thickness of the sphenoid bone (n=176), plasma concentrations of ACTH measured by RIA (n=59) and IRMA (n=112), plasma concentrations of cortisol (n=165), α-MSH (n=141), GH (n=103), PRL (n=78), LH (n=79), TSH (n=38) and T_4 (n=41). These indicators with a probability value less than 0.10 in the univariate analysis were entered into a stepwise multivariate Cox's proportional-hazard analysis with backward elimination using Newton Raphson algorithm.[19]

The UCCR in the time period 6-10 weeks after surgery was measured in 91 of the dogs that went into remission. These dogs were divided into three groups based on the postoperative UCCR; $< 1 \times 10^{-6}$ (37 dogs), 1 to $< 5 \times 10^{-6}$ (35 dogs), 5 to $< 10 \times 10^{-6}$ (19 dogs). Differences between Kaplan-Meier curves of these 3 groups were tested for significance ($P < 0.05$) by the Log Rank test. [19]

Further evaluation of the prognostic factors was performed with SPSS (SPSS Benelux BV, Gorinchem, The Netherlands) statistical package. Comparisons between two groups of dogs were performed with non-parametric tests for independent variables (Mann-Whitney test) and bivariate correlations. Chi-square analysis was used to compare height/width ratio in dogs with remission and residual disease. A P-value < 0.05 was considered significant. Bonferoni correction with factor 3 was applied in case of multiple comparisons. Box-plot graphs were made in Sigma.Plot version 9.0 (Systat Software GmbH, Erkrath, Germany).

Results

The median and range for preoperative variables are presented in Table 1. The median follow-up time was 636 days (range, 1-3002 days). Of the 181 dogs, there were 14 (7.7%) postopera-

tive mortalities, 12 (6.6%) dogs had residual disease and 155 (85.6%) went into remission. Of the dogs in remission, disease recurred in 36 cases (23%).

Table 1
Summary of preoperative variables*

Variable	No.	Median	Range
Age (years)	181	9	3 - 14
Body Weight (kg)	181	15	4 - 61
Pituitary height (mm)	181	5.4	2.1 - 15
Pituitary width (mm)	181	6.2	2.9 - 17.4
Pituitary length (mm)	178	6.0	2 - 18
Pituitary height-to-width ratio	181	0.86	0.48 – 1.7
Pituitary height-to-length ratio	178	0.97	0.48-2.3
Pituitary width-to-length ratio	178	1.1	0.57-2.4
P/B ratio (mm^{-1})	180	0.33×10^{-2}	$0.15\text{-}1.1 \times 10^{-2}$
Pituitary volume (mm^3)	178	89	10-2400
Sphenoid bone thickness (mm)	176	5.0	1.7-10.2
Basal plasma hormone concentrations			
ACTH(RIA) (pmol/l)	59	34	4.4-133
ACTH(IRMA) (pmol/l)	112	19	0.66-156
Cortisol (nmol/l)	165	211	2.4-1300
α-MSH (pmol/l)	141	12	1.4-560
GH (μg/l)	103	0.70	0.10-2.3
PRL (μg/l)	78	8.2	0.80-24
LH (μg/l)	79	5.8	1.0-66
TSH (μg/l)	38	0.14	0.0-1.4
T4 (nmol/l)	41	14	3.0-26

* No. = number of dogs; P/B ratio = pituitary height-to-brain area ratio; ACTH = adrenocorticotropic hormone; RIA = radioimmunoassay; IRMA = two-site immunoradiometric assay; α-MSH = α-melanocyte-stimulating hormone; GH = growth hormone; PRL = prolactin; LH = luteinizing hormone; TSH = thyroid stimulating hormone; T4 = thyroxin

The pituitary gland was not enlarged (P/B ≤ 0.31) in 78 cases, remission occurred in 68 cases (87%) of which disease recurred in 12 cases (18%). The pituitary gland was enlarged (P/B > 0.31) in 102 dogs, of which 86 (84%) went into remission and 24 (28%) had a recurrence.

The plasma α-MSH concentration was < 36 pmol/l in 122 dogs. Of the 19 dogs with elevated α-MSH concentration, 9 had a recurrence. These dogs were all among 11 dogs with preoperative α-MSH concentration > 120 pmol/l. Six of these 9 dogs had dexamethasone-resistant PDH and an enlarged pituitary gland.

The 1-year estimated survival rate was 86% (95% confidence interval [CI], 80 -91%). The 2-year estimated survival rate was 83% (CI, 76-88%), the 3-year estimated survival rate was 80% (CI, 73-86%). The 4-year estimated survival rate was 79% (CI, 70-85%) (Figure 3A). The 1-year estimated disease-free fraction was 90% (CI, 84-94%). The 2-year estimated disease-free fraction was 77% (CI, 68-84%). The 3-year estimated disease-free fraction was 72% (CI, 62-80%). The 4-year estimated disease-free fraction was 62% (CI, 49-72%) (Figure 3B).

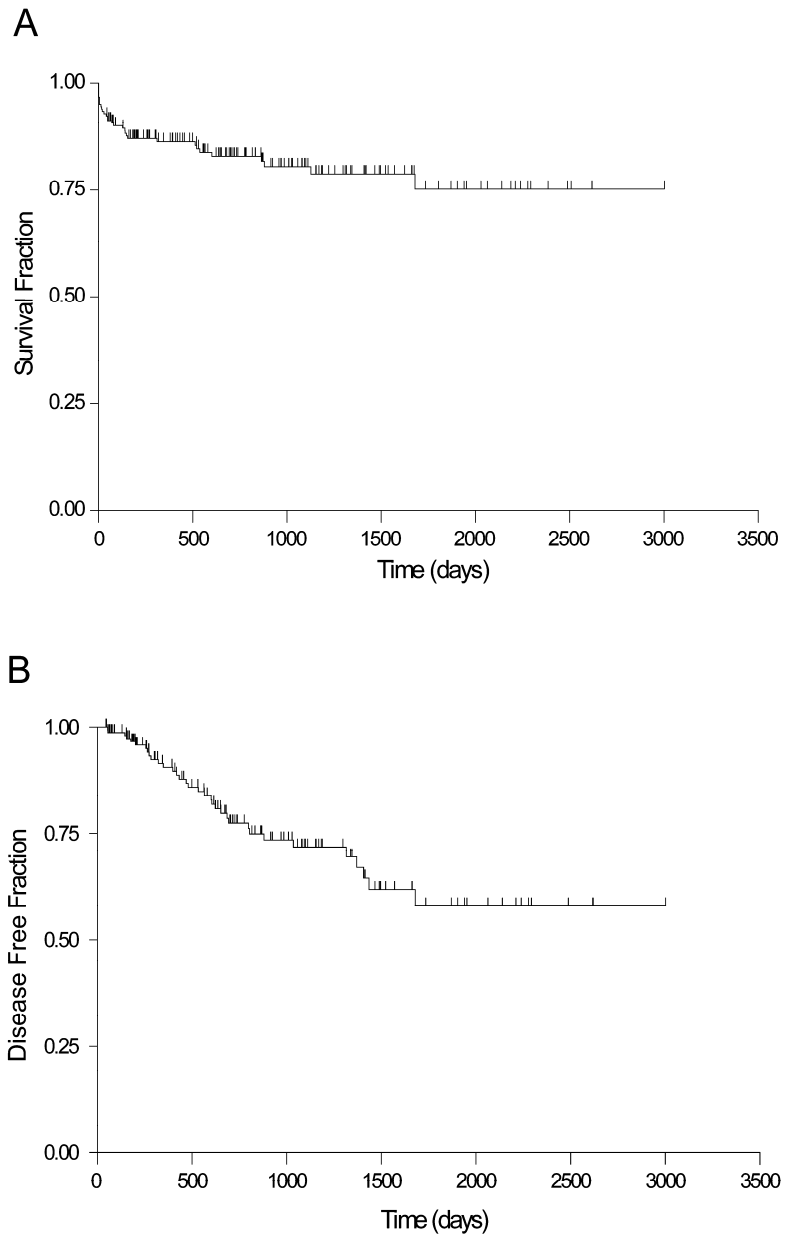

Figure 3. Survival curves calculated with Kaplan-Meijer estimate procedure for dogs after transsphenoidal surgery for the treatment of pituitary-dependent hyperadrenocorticism. (A) Survival time for the 181 dogs included in the study. (B) Disease-free period for 155 dogs with initial remission. Censored cases (still alive at the time of follow-up or died due to unrelated causes) are represented by vertical bars.

Postoperative Mortality

The 14 dogs that died within 4 weeks postoperatively had a median P/B ratio of 0.36 (range, 0.21-0.70), and eight of these dogs had an enlarged pituitary. Two dogs developed hemorrhage from the arterial cerebral circle. Two dogs died within 6 h after surgery, and postmortem examination in one revealed thromboendocarditis of the right atrium, concentric myocardial hypertrophy of the left ventricle and lung edema due to circulatory failure. Four dogs in which surgery was uneventful died one day after surgery; 2 dogs became dyspnoeic, 1 dog had glucocorticoid associated myotonia as was diagnosed preoperatively by electromyography, and 1 dog developed hypernatremia due to insufficient oral fluid intake. Two dogs died 5 days after surgery; one due to accidental IV injection of oral potassium solution, and one, in which surgery and recovery were uneventful, died suddenly at home with unknown cause. Two dogs had a prolonged stay in the intensive care unit for 2 weeks because of severe hypernatremia and diabetic ketoacidosis, and were eventually euthanized. One dog developed severe bronchopneumonia, and died 4 weeks after surgery. One dog was euthanized 16 days after surgery because of perforative peritonitis caused by a foreign body.

Residual Disease

In the 12 dogs with residual disease after surgery the median P/B ratio was 0.40 (range, 0.20-0.76), and eight dogs had an enlarged pituitary. Of the dogs with residual disease, 5 were euthanized or died within 5 months after surgery for reasons associated with hyperadrenocorticism, 2 dogs were euthanized because of aggressive behavior and epileptic seizures 26 and 34 months after surgery, one dog died suddenly at home 2 months after surgery and 4 dogs were still alive at the time of assessment with survival times of 12, 16, 17 and 32 months. Three of the 12 dogs with residual disease were treated with mitotane 3 to 6 months after surgery. At the time of assessment, survival times of these 3 dogs were 17, 26, and 32 months. Bilateral adrenalectomy was performed in one case, and this dog survived for 34 months until it developed seizures and was euthanized. In two dogs, at 5 weeks and 6 months after pituitary surgery, medical treatment with trilostane, a 3 β–hydroxysteroid dehydrogenase inhibitor, was initiated and the survival times were 12 and 16 months.

Remission

Of the 155 dogs in remission, 119 (77%) remained in remission. The median P/B ratio of 118 of these 119 dogs was 0.32 (range, 0.15-1.1) and the pituitary was enlarged in 63 cases (53%). The P/B ratio was not available for one dog. Of the 119 dogs that remained in remission, UCCR was measured 6 to 10 weeks post surgery in 78 dogs. Median basal UCCR at this time was 0.8×10^{-6} (range, $0.2\text{-}9 \times 10^{-6}$). In 38 dogs the UCCR was $< 1 \times 10^{-6}$, in 31 dogs 1 to $< 5 \times 10^{-6}$, and in 9 dogs 5 to $< 10 \times 10^{-6}$. Over time, 69 of the 119 dogs in remission died (10 dogs) or were euthanized (59 dogs) for non-related causes after a median interval of 28 months (range, 1.5-100 months).

Recurrence

Of the 155 dogs in remission, disease recurred in 36 cases (23%) after a median of 16 months (range, 1.8-56 months). The median P/B ratio of these 36 dogs was 0.41 (range, 0.19-0.71) and the pituitary was enlarged in 24 cases (67%). The median UCCR measured 6 to

10 weeks post surgery (in 22 dogs) was 3.0 x 10^{-6} (range, 0.2-8.7 x 10^{-6}). The postoperative UCCR was < 1 x 10^{-6} in 5 cases, 1 to < 5 x 10^{-6} in 8 cases and 5 to < 10 x 10^{-6} in 9 cases. After recurrence of hyperadrenocorticism, 16 cases were treated with mitotane and 5 cases with trilostane. At the time of assessment, 16 dogs were euthanized or had died because of recurrent signs of hyperadrenocorticism, 13 dogs had died because of old age (1 dog), heart failure (1 dog), lung edema (1 dog), neurological signs (1 dog), hind limb weakness (1 dog), drowning (1 dog), chronic nasal bacterial infection (1 dog), thyroid carcinoma and sepsis (1 dog), gastrointestinal symptoms (1 dog), uncontrolled diabetes insipidus (1 dog) and unknown cause (3 dogs). Seven dogs were still alive at time of assessment.

Prognostic Factors

In the univariate Cox's proportional-hazard analysis for survival time of the 181 dogs, the following factors had P-values < 0.10: age, pituitary height, width and length, maximum pituitary dimension, pituitary height-to-width ratio, P/B ratio, pituitary volume, and basal plasma concentration of ACTH(IRMA) (Table 2).

Table 2

Variables with P < 0.10 in univariate Cox's proportional-hazard analysis for survival and disease-free periods after transsphenoidal hypophysectomy for the treatment of pituitary-dependent hyperadrenocorticism in dogs*

Variable	No.	P	HR	95% CI
Survival period				
Age	181	0.030	1.211	1.018-1.440
Pituitary height	181	0.004	1.191	1.057-1.342
Pituitary width	181	0.008	1.163	1.040-1.300
Pituitary length	178	0.001	1.177	1.067-1.298
Max. pituitary dimension	178	0.001	1.182	1.067-1.310
Pituitary volume	178	0.005	1.001	1.000-1.002
Pituitary height-to-brain area ratio	180	0.009	12.82	1.912-85.95
Plasma ACTH (IRMA) conc.	112	0.014	1.003	1.001-1.005
Disease-free period				
Body weight	155	0.049	1.027	1.000-1.054
Body weight group		0.020	(Termwise Wald Test)	
Dogs > 30 kg	0.005	3.344	1.426-7.840	
Mean preoperative UCCR	155	<0.001	1.004	1.002-1.007
Preoperative UCCR (dex)	155	0.005	1.004	1.001-1.007
Pituitary height	155	<0.001	1.311	1.150-1.494
Pituitary width	155	<0.001	1.273	1.129-1.437
Pituitary length	152	<0.001	1.263	1.125-1.418
Max. pituitary dimension	152	<0.001	1.301	1.156-1.463
Pituitary height-to-width ratio	155	0.093	4.236	0.7851-22.86
Pituitary volume	152	<0.001	1.002	1.001-1.003
Pituitary height-to-brain area ratio	154	<0.001	45.62	5.930-351.0
Thickness of the sphenoid bone	150	0.002	1.360	1.123-1.647
Plasma α-MSH conc.	120	0.024	1.002	1.000-1.004

* No. = number of dogs; HR = Hazard Ratio; CI = Confidence Interval; Max = maximum; conc. = concentration; ACTH = adrenocorticotropic hormone; IRMA = two-site immunoradiometric assay; UCCR= Urinary corticoid-to-creatinine ratio; UCCR (dex) = UCCR after high-dose dexamethasone suppression; α-MSH = α-melanocyte-stimulating hormone (CI, 71-95%)

In the multivariate analysis, high age and increased pituitary length were associated with reduced survival time after hypophysectomy, when ACTH(IRMA) was not included. If ACTH(IRMA) was added in a new multivariate analysis, high age and high plasma ACTH (IRMA) concentration were associated with reduced survival time after hypophysectomy (Table 3).

Table 3
Multivariate Cox's proportional-hazard analysis (backward using Newton Raphson algorithm) for survival and disease-free periods after transsphenoidal hypophysectomy for the treatment of pituitary-dependent hyperadrenocorticism in dogs*

Time period	No.	No. (events)	Variables entered	Significant variables	P	HR	95% CI
Survival	178	32	age, pituitary length, P/B ratio	age pituitary length	0.0187 < 0.001	1.221 1.206	1.034-1.443 1.081-1.345
Survival	112	19	age, pituitary length, P/B ratio, plasma ACTH(IRMA) conc.	age Plasma ACTH (IRMA) conc.	0.088 0.039	1.205 1.003	0.9723-1.493 1.000-1.005
Disease-free	152	35	body weight, mean preop. UCCR, preop UCCR (dex), pituitary length, P/B ratio, sphenoid bone thick.	mean preop. UCCR sphenoid bone thick. P/B-ratio	0.017 0.032 0.025	1.004 1.241 17.22	1.001-1.007 1.018-1.512 1.422-208.4
Disease-free	117	27	body weight, mean preop. UCCR, preop. UCCR (dex), pituitary length, P/B ratio, sphenoid bone thickness plasma. α-MSH conc.	mean preop UCCR sphenoid bone thickness Plasma α-MSH conc.	0.070 0.022 0.030	1.004 1.309 1.002	0.9997-1.008 1.039-1.649 1.000-1.004

* No. = number of dogs; HR = hazard ratio; CI = confidence interval; P/B-ratio = pituitary height-to-brain area ratio; conc. = concentration; ACTH = adrenocorticotropic hormone; IRMA = two-site immunoradiometric assay; preop. = preoperative; UCCR= urinary corticoid-to-creatinine ratio; UCCR (dex) = UCCR after high-dose dexamethasone suppression; thick. = thickness; α-MSH = α-melanocyte-stimulating hormone

In the univariate Cox's proportional-hazard analysis for disease-free fraction in 155 dogs that went into remission, the following variables had a P-value < 0.10: body weight, body weight group, mean preoperative UCCR, UCCR after dexamethasone suppression, pituitary height, width and length, maximum pituitary dimension, pituitary volume, P/B ratio, thickness of the sphenoid bone, and basal plasma α-MSH concentration (Table 2). In the multivariate analysis the mean preoperative UCCR, P/B ratio and thickness of the sphenoid bone were associated with an increased risk for recurrence of hyperadrenocorticism when α-MSH was not included. If α-MSH was added to the multivariate analysis, the mean preoperative UCCR, thickness of the sphenoid bone and the plasma α-MSH concentration were associated with an increased risk for recurrence of hyperadrenocorticism (Table 3).

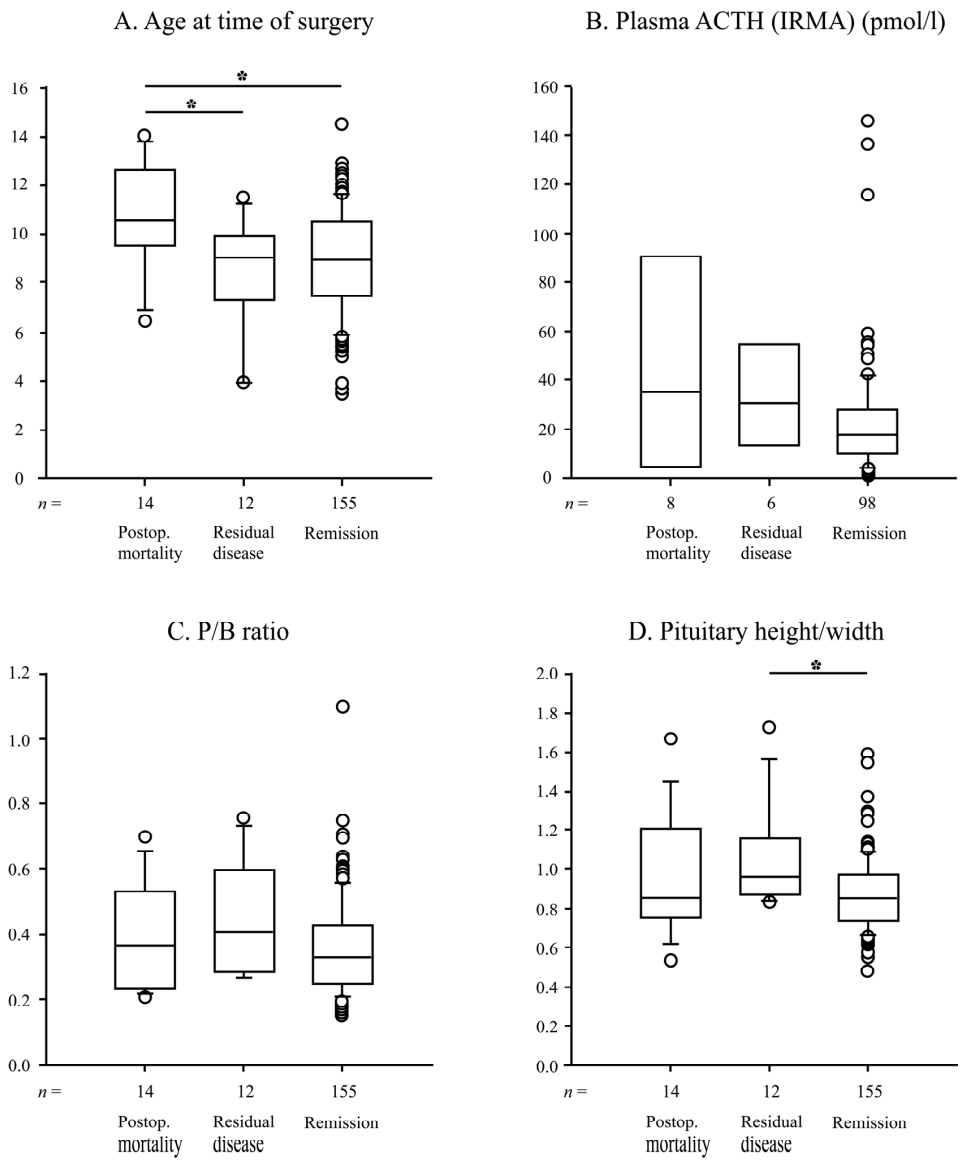

Figure 4. Box plot graphs over distribution of (A) age at time of surgery, (B) basal plasma ACTH concentration measured with a highly specific two-site immunoradiometric assay (IRMA), (C) pituitary height-to-brain area ratio (P/B ratio) and (D) pituitary height-to-width ratio in cases with early postoperative mortality (< 4 weeks), dogs with residual disease and dogs in remission. Significant differences between groups (connected with a line) are indicated with asterices. *P <0.05

The dogs with postoperative mortality were significantly older than the dogs in remission ($P < 0.05$) and than the dogs with residual disease after surgery ($P < 0.05$) (Figure 4A). The P/B ratio and basal plasma ACTH(IRMA) concentration were not different in the dogs with postoperative mortality, residual disease or remission (Figure 4B,C). In the 12 cases with residual disease, the pituitary height-to-width ratio was significantly ($P < 0.05$) higher than in those that went into remission (Figure 4D).

Postoperative UCCR was measured 6 to 10 weeks after hypophysectomy in 91 dogs. In 37 of these 91 dogs the UCCR was $< 1 \times 10^{-6}$; and the estimated 1-year disease-free fraction was 97% (CI, 82-100%), the estimated 2-year disease-free fraction was 88% and the estimated 3-year disease-free fraction was 82% (CI, 62-93%). In 35 dogs the postoperative UCCR was 1 to $\leq 5.0 \times 10^{-6}$ and the estimated 1-year disease-free fraction was 97% (CI, 81-100%) and the estimated 2-year disease-free fraction was 78% (CI, 56-90%). In 19 dogs the postoperative UCCR was 5 to $\leq 10 \times 10^{-6}$ and the estimated 1-year disease-free fraction was 65% (CI, 34-84%), and the estimated 2-year disease-free fraction was 40% (CI, 15-65%).When these 3 groups were compared, the disease-free fractions were significantly lower in the group of dogs with a postoperative UCCR of 5 to $\leq 10 \times 10^{-6}$ than those in the groups with a postoperative UCCR of $< 5 \times 10^{-6}$ (Figure 5). In the univariate Cox's proportional-hazard analysis for disease-free fraction, dogs with a postoperative UCCR of 5 to 10×10^{-6} had a significantly (P=0.001) higher risk of having a recurrence (HR 7.088; CI, 2.361-21.280) than dogs with a postoperative UCCR of $< 1 \times 10^{-6}$.

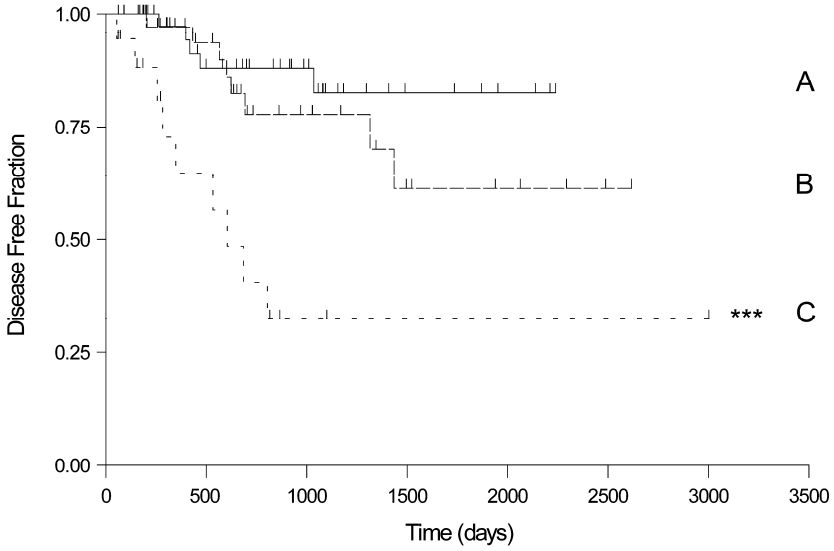

Figure 5. Survival curves calculated with Kaplan-Meijer estimate procedure for disease-free period in dogs with postoperative 6 to 10 week urinary corticoid-to-creatinine ratios (A) $< 1 \times 10^{-6}$, (B) 1 to $\leq 5.0\text{-x } 10^{-6}$, and (C) 5 to $\leq 10 \times 10^{-6}$ after transsphenoidal hypophysectomy for the treatment of pituitary-dependent hyperadrenocorticism in dogs. ***P < 0.001 compared with A and B

Discussion

The results of this study demonstrate that increased age, pituitary size, and basal plasma ACTH concentration are risk factors for shorter postoperative survival times, and that the thickness of the sphenoid bone, mean preoperative UCCR, pituitary size and plasma α-MSH concentration are risk factors for recurrence after transsphenoidal hypophysectomy for the treatment of PDH in dogs. Hypophysectomy in dogs has a higher postoperative mortality (8%), but remission (86%) and recurrence rates (23%) are comparable with those reported for humans with Cushing's disease.[2,7,41,45,49] The total success-rate in this study is 67% which is comparable to what has been reported in humans after pituitary surgery when long-term recurrences are considered.[2] The study-period of 12 years equals approximately 70-80 years in humans. Thus, we could include late recurrences. Recurrences despite the aim of complete hypophysectomy probably has to be ascribed to remnant islets of pituitary cells, as previously shown by Meij and co-workers.[36] These remnant cells may be of neoplastic origin or representants of unaffected pituitary cells. Regrowth of remnant adenomatous corticotrope cells most probably is responsible for part of the recurrences reported in the present study.[23] However, the very late recurrences (> 3-4 years after surgery) may very well be the result of de novo formation of adenomatous tissue from remnant corticotropes.

Prognosticators for Survival Time

The survival time after surgery is influenced by external factors, for example decision of the owners for either medical treatment or euthanasia in case of recurrence. The analysis of survival time also includes cases with postoperative mortality and residual disease, which are excluded from the analysis of disease-free period due to the absence of initial remission. This explains the initially surprising finding that age was a prognosticator for survival time. The Kaplan-Meijer analysis compensates for age by censoring deaths that are not related to the disease, therefore, the prognosticator age had to be related to PDH or to exert its effect in the early postoperative period (within 4 weeks after surgery) in which all deaths were defined as (disease-related) events. In line with the latter explanation, dogs that died during the first 4 weeks after surgery were significantly older than the dogs with residual disease or the dogs that achieved remission. Subsequently, the Cox's proportional-hazard analysis of survival time identified age as a risk factor for early mortality, which could be ascribed to the higher age among the dogs with immediate postoperative mortality. Whether the higher postoperative mortality in older dogs is caused by the presence of concurrent unrelated disease or a reflection of higher risk of complications due to a longer preoperative exposure to hypercortisolism remains to be investigated. For example, human patients with Cushing's disease comprise a high-risk group for pre- and postoperative morbidity and mortality of cardiovascular events and thromboembolism.[9,33,49]

In agreement with our previous study[24] and reports on transsphenoidal surgery for the treatment of Cushing's disease in humans,[11,13,41,55,60] it was expected to find pituitary size as a prognosticator for survival time. The present study provides calculated hazard ratios of the influence of the pituitary size on surgical outcome, which can be applied for individual risk calculations. There was no difference in pituitary size among dogs with postoperative mortality, residual disease or remission. Thus, in contrast to age, the pituitary size influences the survival time beyond the postoperative 4-week period. Human patients with Cushing's disease and suprasellar extension of the pituitary tumor[22] and tumor invasion into the

cavernous sinus [6,11] have higher risk of residual disease. In this study dogs with residual disease had higher pituitary height-to-width ratio than dogs that went into remission.

Interestingly, preoperative plasma concentration of ACTH(IRMA) was a significant prognosticator for survival time, and replaced the pituitary length in the final equation of the multivariate analysis. High preoperative basal plasma ACTH concentrations have been reported in humans with residual disease after transsphenoidal surgery,[11,49] and this has been ascribed to the large tumor size in this subgroup. However, low plasma ACTH concentrations have also been reported in humans with large pituitary tumors.[26] There was no significant correlation between pituitary size and the basal plasma ACTH(IRMA) concentration in the present series of dogs. However, in previous reports on dogs,[10,21] the basal plasma ACTH concentration correlated with pituitary size, but in this case the ACTH was determined by the RIA that also measures ACTH-precursor peptides. It is therefore concluded that plasma ACTH concentration, besides being related to adenoma size, may also give additional information on intrinsic characteristics of the corticotrope associated with poorer surgical outcome.

Prognosticators for Disease-Free Period (Recurrences)

It may be hypothesized that prognostic factors for recurrences are associated with increased risks of leaving remnant pituitary adenoma tissue in the pituitary fossa. In the dog, there is a large difference in skull size between breeds. For example, the smallest dog operated on in this study was a Yorkshire Terrier with a body weight of 4 kg and the largest dog was an Alaskan Malamute with a body weight of 61 kg. The differences in skull sizes and shape affect the distance between the surgeon and the pituitary fossa and consequently the visibility of the surgical field. An even more important factor for the accessibility of the surgical field may be the thickness of the sphenoid bone. In the future, this risk factor may be better addressed with the use of image-guided endoscopy which has gained popularity in pituitary surgery in human.[27,42] Using a rigid endoscope to explore the pituitary fossa for pituitary remnants after hypophysectomy, the recurrence rate may be reduced in the long term.

The pituitary size has frequently been reported to influence surgical outcome,[13,24,41,55] and it may be hypothesized that the larger the tumor, the higher the risk of remnant adenoma cells in the fossa. However, when added to the multivariate analysis, the basal plasma α-MSH concentration replaced the P/B ratio in the multivariate equation. This finding can partly be explained by the correlation between basal α-MSH concentration and pituitary size, as previously published for dogs.[10] Additionally, the basal α-MSH concentration may reflect an aggressive behavior of the pituitary tumor, similarly to what has been described for high plasma pro-opiomelanocortin concentrations in humans with aggressive corticotrope cell tumors.[48] In the dog, the plasma α-MSH concentration correlates significantly with plasma ACTH precursor concentrations.[10] Interestingly, disease recurred in most of dogs with high α-MSH concentrations. These dogs usually had dexamethasone resistant PDH and enlarged pituitaries, most of them probably of pars intermedia origin.[51,52]

Independent of pituitary size and the plasma α-MSH concentration, a high preoperative UCCR was associated with increased risk of recurrence in dogs, which is in agreement with a study in humans where a high urinary cortisol excretion was a risk factor for recurrence.[57] Measuring cortisol in morning urine has the advantage that it mirrors the integrated production over a period of about 8 h, and thereby adjusts for the wide and rapid fluctuations in plasma cortisol levels. It has been speculated that the degree of resistance to suppression of

the cortisol secretion by dexamethasone correlates with the risk of recurrences.[11] In the present study, after high-dose dexamethasone administration, the relative suppression of pre-operative UCCR was not a prognosticator for disease-free period, but the absolute suppressed UCCR-value was.

Within the group of corticotrope cell tumors there is a wide heterogeneity with regard to cellular characteristics. The results of this study indicate that measurements of the plasma α-MSH and ACTH concentrations and urinary cortisol may contribute to pre-operative characterization of the pituitary lesion.

Postoperative UCCR

One may hypothesize that dogs with postoperative UCCRs in the upper normal range have a higher risk of recurrence or even can be considered to have residual disease. In the present study the postoperative UCCRs sampled 6-10 weeks post surgery were analyzed. In this time period remnant islets of pituitary tissue may release ACTH in response to CRH stimulation.[37] Indeed, as confirmed in the present study, there is a considerable difference in the remission rate between dogs with a low UCCR ($< 5 \times 10^{-6}$) 6 to 10 weeks post surgery compared to dogs with UCCR between 5 and 10×10^{-6}. However, a urinary cortisol below detection limits ($UCCR < 1 \times 10^{-6}$) is no guarantee for lifelong remission, whereas dogs with cortisol secretion in the upper normal range do not all develop a recurrence. These findings further illustrate the difficulty of distinguishing remnant normal corticotropes from remnant adenoma cells, as described previously for dogs.[36,37] The same holds true for selective pituitary adenomectomy in humans with Cushing's disease.[2,12,17,45,49,54,66] It should be noted that the dogs in the present study were on a physiological dose of hydrocortisone, which was withheld for 12 h prior to urine sampling. In humans, the best predictive values for surgical outcome were achieved when no cortisol was administered, until symptoms of hypocortisolism arose combined with early postoperative cortisol measurement and dexamethasone suppression test.[28,54]

Conclusions

We have identified several risk factors that can be addressed to further improve surgical outcome after hypophysectomy in the treatment of PDH in dogs. Old age, large pituitary size and high basal plasma ACTH concentration are significant prognosticators for postoperative survival. Pituitary enlargement, elevated plasma α-MSH concentration, thick sphenoid bone and mean preoperatively UCCR are significant risk factors for recurrence. In parallel with findings in human studies, postoperative UCCR measured at 6 to 10 weeks after surgery can be used as guidance for predicting surgical outcome, albeit it is insufficient to predict every case of recurrence.

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CHAPTER 5

Plasma profiles of adrenocorticotropic hormone, cortisol, α-melanocyte stimulating hormone, and growth hormone in dogs with pituitary-dependent hyperadrenocorticism before and after hypophysectomy

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Abstract

The 6-hour plasma profiles of adrenocorticotropic hormone (ACTH), cortisol, α-melanocyte stimulating hormone (α-MSH), and growth hormone (GH) were studied in 17 dogs with pituitary-dependent hyperadrenocorticism (PDH) before and after hypophysectomy. The aim of the study was to investigate the relation between the hormone profile charateristics and recurrence of PDH after surgery.

The four hormones were secreted in a pulsatile fashion. The basal plasma cortisol concentration and area under the curve (AUC) for cortisol were significantly higher in the PDH cases than in 8 controls. The characteristics of the plasma profiles of ACTH and α-MSH were not significantly different between the PDH cases and the controls. In the PDH cases, less GH was secreted in pulses than in the controls, but the difference was not significant The basal plasma cortisol concentration, the AUC for ACTH and cortisol, and the pulse frequency of ACTH and cortisol decreased significantly after hypophysectomy for the group of PDH cases. The basal plasma concentrations of ACTH and α-MSH, the AUC for α-MSH and the characteristics of the plasma GH profiles of the PDH cases remained unchanged after hypophysectomy. No pulses of α-MSH were observed after hypophysectomy. The co-occurrence between the ACTH and cortisol pulses decreased significantly with hypophysectomy. The postoperative pulse frequency of ACTH was the only characteristic with predictive value for recurrence of PDH after hypophysectomy

The results of this study demonstrate that ACTH, cortisol, α-MSH, and GH are secreted in a pulsatile fashion in dogs with PDH. Hypophysectomy effectively reduces the secretion of ACTH and cortisol. The presence of ACTH pulses after hypophysectomy is a risk factor for recurrence of hyperadrenocorticism.

Pituitary-dependent hyperadrenocorticism (PDH) is a common spontaneous endocrine disorder in dogs which shows many similarities with Cushing's disease in humans.[16] As in humans with Cushing's disease,[24,42] PDH in dogs is characterized by adrenocorticotropic hormone (ACTH)-induced hypercortisolism and reduced sensitivity to glucocorticoid feed-back inhibition,[3,45] in most cases with preserved ability to respond to corticotrophin releasing hormone (CRH) stimulation.[27,42] The excessive pituitary secretion of ACTH originates from a corticotropic adenoma in the pars distalis or the pars intermedia of the adenohypophysis. PDH caused by an adenoma in pars intermedia is often characterized by highly elevated plasma concentrations of α-melanocyte stimulating hormone (α-MSH) and strong resistance to dexamethasone suppression.[3,38,43] In Cushing's disease there are not only alterations in ACTH secretion but also in the secretion of other pituitary hormones.[47,50,55] In dogs with PDH these changes may include basal levels and response to stimulation,[27] as well as the pulsatile release pattern that is characteristic of pituitary hormone secretion.[4,6,18,19,21,22,26] For example, plasma growth hormone (GH) concentration and its responsiveness to stimulation with GH-releasing hormone (GHRH) have been reported to be decreased in dogs with PDH, most likely due to glucocorticoid-induced alterations in the function of pituitary somatotropic cells and changes in supra-pituitary regulation.[27] In addition, canine PDH is associated with less GH secreted in pulses than in control dogs.[26]

Pituitary surgery is the treatment of choice in humans with Cushing's disease.[41] Preferrably an adenomectomy is performed, but 15-26% of the cases undergo a total hypophysectomy.[2] Transsphenoidal hypophysectomy is an effective treatment in dogs with PDH.[30] In both species recurrence of the disease is a serious problem. In a study on 150 dogs with PDH, the fraction that relapsed within two years after hypophysectomy was 25%.[12] In humans the recurrence rate is 5-30% after pituitary surgery for corticotropic adenomas.[37,41]

After transsphenoidal hypophysectomy in healthy dogs residual pituitary cells have been observed in the pituitary fossa.[1,29,35] Such cells are devoid of the direct influence of the hypothalamus due to section of the pituitary stalk. Upon administration of secretagogues, the residual corticotropic cells appeared functional. They secreted ACTH after stimulation with CRH.[28] Stimulation with other secretagogues, e.g., thyroptropin releasing hormone (TRH), GHRH, and gonadotropin-releasing hormone (GnRH), evoked no adenohypophyseal hormone secretion.[29] At 8-weeks after surgery, the CRH stimulation test was not able to identify the dogs that would later relapse.[28] The pulsatile secretion pattern of remnant pituitary cells has not yet been studied. It was hypothesized that the secretion pattern would differ between remnant adenomatous and normal corticotropic cells and that the profile characteristics after hypophysectomy would hold predictive value for recurrence of hyperadrenocorticism.

Here we report on the 6-hour plasma profiles of ACTH, cortisol, α-MSH, and GH in dogs with PDH before and after transsphenoidal hypophysectomy. The aim of the study was to investigate the relation between the hormone profile characteristics and recurrence of PDH after surgery.

Materials and Methods

Animals, diagnosis, and treatment of PDH

Seventeen dogs of different breeds with PDH were included in the study. The group comprised 2 Miniature Poodles, 1 Dachshund, 1 Basset Fauve de Bretagne, 1 Cairn Terrier, 1 English Cocker Spaniel, 1 Pitt Bullterrier, 1 Shetland Sheepdog, 1 Soft Coated Wheaten Terrier, 1 Standard Poodle, 1 Welsh Corgi and 6 cross bred dogs. There were 6 female (2 spayed) and 11 male (2 castrated) dogs with a median age of 8 years (range, 5-12 years), and a median body weight of 15 kg (range, 7-27 kg).

The diagnosis of hyperadrenocorticism was based upon averaged urinary corticoid-to-creatinine ratios (UCCR) in two consecutive morning urine samples. In all animals the UCCRs (median, 44 x 10^{-6}; range, 23-321 x 10^{-6}) exceeded the ratios found in 87 healthy companion dogs (range 0.3-8.3 x 10^{-6}).[54] After collection of the second urine sample, three oral doses of 0.1 mg dexamethasone per kg body weight were administered at 8-h intervals. In 13 cases the UCCR in the third sample was less than 50% of the mean of the first two samples, and PDH was diagnosed.[9] In 4 cases with less than 50% suppression of the third UCCR, pituitary dependency was secured by measurements of plasma ACTH concentrations and further supported by visualization of the adrenals by ultrasonography and by pituitary imaging with computed tomography (CT).[3,44,51,52]

Transsphenoidal hypophysectomy was performed according to a microsurgical technique described previously.[31] Postoperative care and hormone supplementation were according to previously published protocols.[12,30,31] Briefly, hydrocortisone and desmopressin were directly administrated after the removal of the pituitary gland. When the dogs had resumed drinking and eating, oral substitution therapy was started with cortisone acetate and thyroxine. The dose of cortisone acetate was gradually lowered over a period of 4 weeks to a physiological dose. Desmopressin was administered for 2 weeks routinely and continued if polyuria due to central diabetes insipidus persisted.

Re-examination after 8 weeks included physical examination, routine blood chemistry, measurements of basal plasma thyroxine concentration at 10-12 hours after L-thyroxine medication, and basal UCCR in duplicate at 24 hours after cortisone medication (median 1.7 x 10^{-6}; range 0.3-5.2 x 10^{-6}). UCCRs were measured again half a year after surgery and thereafter once a year. In case of suspicion of recurrence, UCCRs were determined earlier. Urine samples were mailed to our laboratory, and follow-up reports were obtained from the above mentioned routine follow-up examinations in the hospital and during telephone conversations with the owner and/or the referring veterinarian.

One dog died in the early postoperative period (within 4 weeks) due to kidney failure. In all 16 remaining dogs there was remission: UCCR < 10 x 10^{-6} and resolution of clinical signs of hyperadrenocorticism. Recurrence was defined as UCCR ≥ 10 x 10^{-6} and/or return of signs and symptoms of hyperadrenocorticism after initial complete remission. This occurred in 9 of the 16 dogs after median 652 days (range, 201-1679 days).

Control dogs for pulsatile secretion

The plasma profiles of cortisol, ACTH, and α-MSH of the PDH cases were compared with those obtained in 8 healthy beagle dogs (4 intact females and 4 intact males, with body weight ranging from 12-25 kg, and age ranging from 2-5 years).[19] The plasma GH profiles of

the PDH dogs were compared with those of plasma obtained in 6 healthy female beagle dogs (with body weight ranging from 12-27 kg, and age ranging from 7-9 years).[26]

Sample collection for pulsatile plasma profile

In the PDH dogs, the 6-hour plasma hormone profiles were determined 2 to 34 days (median, 8 days) before surgery and 50 to 133 days (median, 71 days) after hypophysectomy. Pre-operative 6-hour plasma profiles of ACTH and cortisol were available from 17 cases, of α-MSH from 14 cases, and of GH from 14 dogs. After hypophysectomy, 6-hour plasma profiles of ACTH, cortisol became available from 14 cases, of α-MSH from 13 cases and of GH from 11 cases.

Food but not water was withheld from the animals 12 hours prior to the blood sampling. Blood samples (4 ml) were collected from the jugular vein by an experienced technician at 10-min intervals between 8.00 a.m. and 2.00 p.m. Blood was collected in pre-chilled EDTA-coated tubes on ice, and centrifuged at 4º C (2000g, 10 minutes). Plasma was stored at -20º C until assayed.

This study on the pulsatile secretion was approved by the Ethical Committee of Utrecht University and for the PDH dogs informed consent was obtained from the owners.

Hormone determinations

Plasma ACTH concentration was measured using a commercially available two-site immunoradiometreic assay (IRMA) (Nichols Institute, Wijchen, The Netherlands). The anti-serum was highly specific for ACTH (1-39). A polyclonal antibody was bound specifically to the C-terminal region of ACTH. The radioiodinated monoclonal antibody was bound only to the N-terminal region of ACTH. The intra-assay coefficient of variation (CV) was 3.2%, the inter-assay CV was 7.8%, and the sensitivity was 0.22 pmol/l. There was no cross-reaction between the antiserum and α-MSH or ACTH precursors.[3,40]

Plasma cortisol concentration was measured with a solid phase ^{125}I radioimmunoassay (RIA) (Coat-A-Count® Cortisol, Diagnostic Products Corporation, Los Angeles, USA). The antiserum was highly specific for cortisol, with very low cross-reactivity to other compounds that were present in patient samples. Neither protein, lipemia, bilirubin nor hemolysis had any significant effect on the assay. The intra-assay CV was 4.5-6.3%, the inter-assay CV was 4%, and the sensitivity was 5.5 nmol/l

The urinary corticoid concentration was measured with RIA as described previously.[45] The intra-assay CV was 6%, the inter-assay CV was 8%, and the sensitivity was 1 nmol/l. The urinary corticoid concentration was related to the urinary creatinine concentration (Jaffé kinetic method, initial rate reaction) and the UCCR was calculated.[45,48]

Plasma concentration of α–MSH was measured with RIA without extraction as described previously.[43] The intra-assay CV was 10%, the inter-assay CV was 23%, and the sensitivity was 3 pmol/l. The antiserum had less than 0.1% cross-reactivity with ACTH (1-39) and 4% cross-reactivity with ACTH (1-24).

Plasma GH concentration was measured by a homologous RIA as described previously.[7] The intra-assay CV was 3.8%, the inter-assay CV was 7.2%, and the sensitivity was 0.3 µg/l. The degree of cross-reaction of canine prolactin was 2%.

Data analysis

The 6-hour plasma hormone profiles were analysed using the Pulsar program developed by Merriam and Wachter.[32] The program identifies secretory peaks by height and duration from a smoothed baseline, using the assay standard deviation (SD) as a scale factor. The cut-off parameters G1-G5 of the Pulsar program were set at 3.98, 2.4, 1.68, 1.24, and 0.93 times the assay SD as criteria for accepting peaks 1, 2, 3, 4, and 5 points wide. The smoothing time, a window used to calculate a running mean value, was set at 5 h. The weight assigned to peaks was 0.05. The A, B, and C values of the Pulsar program used to calculate the variance of the assay was set at A=0, B=7.1, and C=32 for ACTH; at A=0, B=4.8, and C=114 for cortisol; at A=0, B=-2.44, and C=451 for α-MSH; and at A=0, B=7.2, and C=5 for GH. The values extracted from the Pulsar analyses included the overall mean of the smoothed baseline, area under the curve (AUC) above the zero level, and the number of significant pulses per 6h.

Differences in variables between control dogs and dogs with PDH were assessed by the non-parametric Mann-Whitney test (with Bonferroni correction). Differences in variables before and after surgery were assessed by Wilcoxon signed ranks test for related samples (with Bonferroni correction). A $P<0.05$ was considered significant. Fishers's exact test was used to analyze the co-occurrence of significant ACTH, cortisol and α-MSH pulses.

Univariate Cox proportional hazard fit analyses were performed using Newton Raphson algorithm for the disease-free period on the characteristics of the plasma hormone profiles (mean of the smoothed baseline, AUC, pulse frequency) for ACTH, cortisol, α-MSH and GH before and after hypophysectomy.

Results

All four hormones were secreted in a pulsatile fashion in control dogs as well as in dogs with PDH (Figure 1a and b). Representative graphs of the plasma profiles for the controls have been published previously.[19,26]

ACTH and cortisol

In the dogs with PDH the basal plasma cortisol concentration ($P<0.005$) and the AUC for cortisol ($P<0.001$) were significantly higher than those in the controls. There was no significant difference in cortisol pulse frequency. After hypophysectomy the basal plasma cortisol concentration ($P<0.005$), the AUC for cortisol ($P<0.005$), and the cortisol pulse frequency ($P<0.005$) decreased significantly (Figure 2). The basal plasma ACTH concentration, the AUC for ACTH, and the ACTH pulse frequency in the PDH dogs were not significantly different from those in the control dogs. Significant ACTH pulses were identified by the Pulsar program in 14 of the 17 dogs with PDH. After surgery the AUC for ACTH ($P<0.05$) and the ACTH pulse frequency ($P<0.05$) decreased significantly, whereas the basal plasma ACTH concentration tended to decrease ($P=0.052$) (Figure 2). Significant ACTH pulses were identified in 10 of the 14 dogs after hypophysectomy, 8 of these 10 dogs had a recurrence later on.

In the PDH dogs and in the control dogs, the majority of ACTH pulses coincided with cortisol pulses. In the control dogs, 23 of 26 significant ACTH pulses identified by the Pulsar program coincided with 21 of 38 significant cortisol pulses. In the PDH dogs, 32 of 36 significant ACTH pulses coincided with 32 of 65 significant cortisol pulses. The difference be-

tween co-occurrence of ACTH and cortisol pulses in control and PDH dogs was not significant. After surgery 5 of 12 significant ACTH pulses coincided with 5 of 26 significant cortisol pulses. The difference between co-occurrence of ACTH and cortisol pulses before and after surgery was significant ($P<0.01$).

α-MSH

The basal plasma α-MSH concentration, the AUC for α-MSH, and the α-MSH pulse frequency in the PDH cases were similar to those in the controls. Significant α-MSH pulses were identified in 7 of the 14 PDH cases. After hypophysectomy no significant α-MSH pulses were detected in any of the dogs. In one dog with dexamethasone-resistant PDH, a very high basal plasma α-MSH concentration (215 pmol/l; reference range ≤ 36 pmol/l)[3,23] was found and 12 significant α-MSH pulses were identified (Figure 1b). In this dog the postoperative basal α-MSH concentration was 4.5 pmol/l (Figure 1b).

In the PDH dogs and in the controls α-MSH pulses frequently coincided with ACTH pulses. In the controls 3 of 4 significant α-MSH pulses coincided with significant ACTH pulses. In the PDH cases 9 of 25 significant α-MSH pulses coincided with significant ACTH pulses. In the dog with 12 significant α-MSH pulses only 3 (high) α-MSH pulses coincided with a significant ACTH pulse (Figure 1b). In some cases, an α-MSH pulse preceded an ACTH pulse.

GH

The basal plasma GH concentration, the AUC for GH, and the GH pulse frequency were not significantly different between controls and PDH dogs, and also not between the PDH cases before and after hypophysectomy. Significant GH pulses were detected in 11 of 15 dogs with PDH. After hypophysectomy significant GH pulses were detected in 8 of 11 PDH cases.

Disease-free period and identification of risk parameters

The median disease-free period was 880 days (95% CI 732-1028 days). The 1-year disease-free fraction was 87% (95% CI 56-96%) and the 2-year disease-free fraction was 65% (95% CI 35-84%). In 9 of the 16 cases, hyperadrenocorticism recurred after 1.5 to 5.5 years. In the univariate Cox proportional hazard analysis there was no association between preoperative hormone values and recurrence of hyperadrenocorticism. Of the values after hypophysectomy, the ACTH pulse frequency was associated with a significant ($P<0.05$) higher risk for recurrence of hyperadrenocorticism (Hazard ratio 5.357; 95% CI 1.003-28.611). A higher AUC for GH after surgery tended to be associated with a lower risk of recurrence ($P=0.076$) (Hazard ratio 0.768, 95% CI 0.553-1.066).

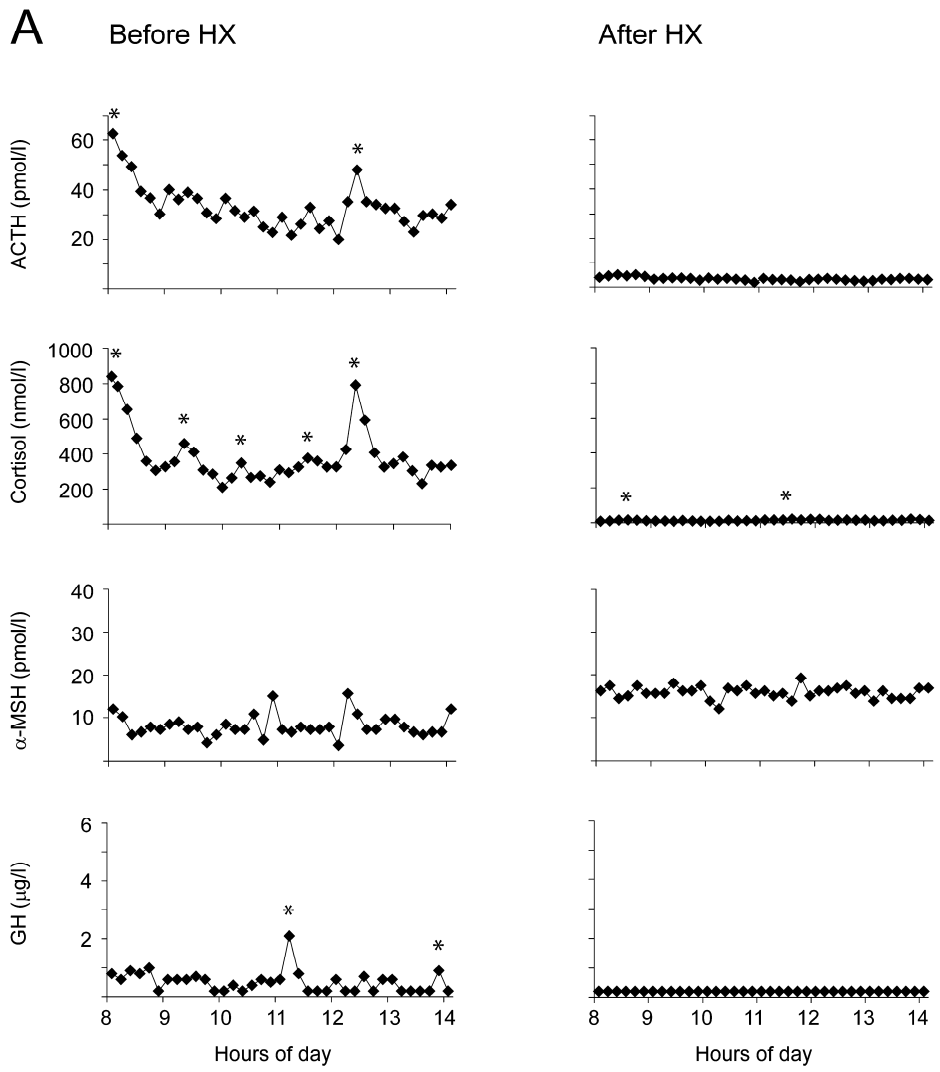
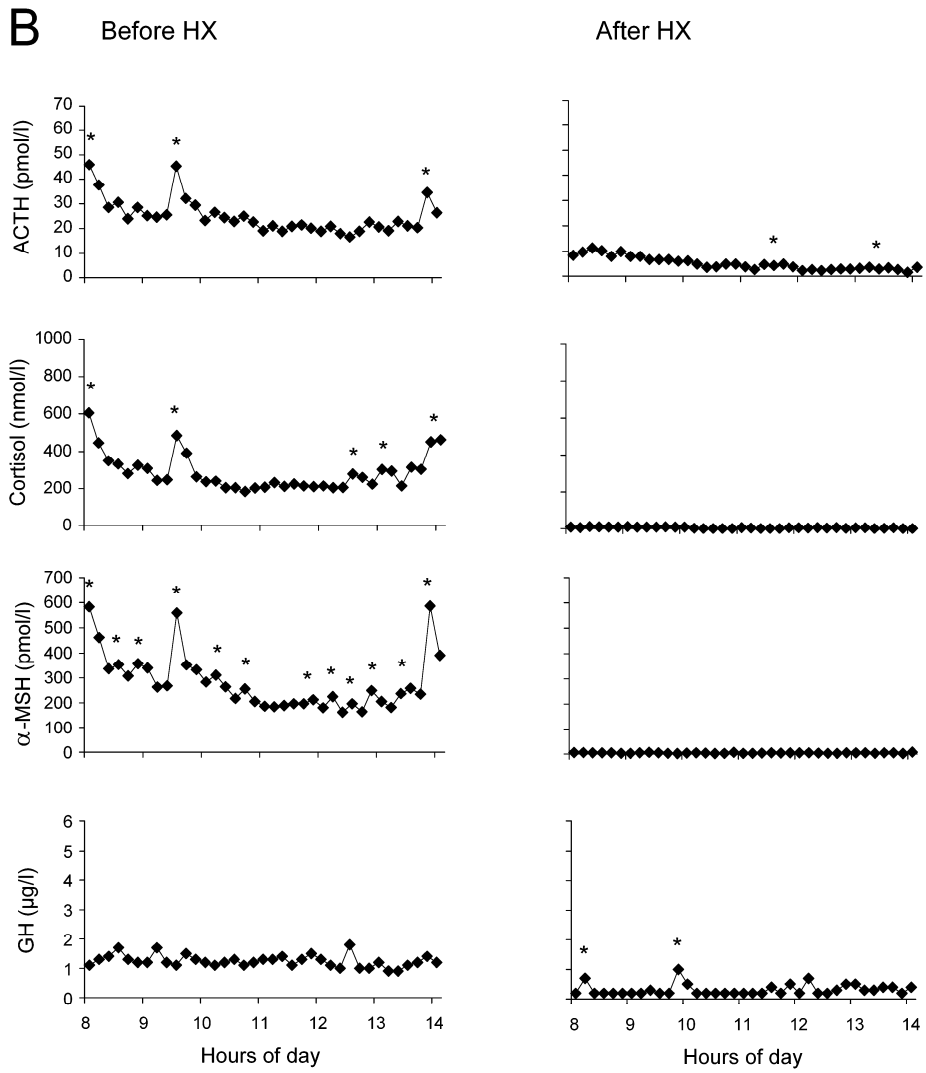


Figure 1. The plasma profiles of ACTH, cortisol, α-MSH, and GH before and after hypophysectomy in (A) a representative dog with pituitary-dependent hyperadrenocorticism (PDH), and (B) a dog with PDH with markedly elevated plasma α-MSH concentrations before hypophysectomy (note difference in scale y-axis. Blood samples were collected at 10-min intervals for 6 hours. Significant pulses, calculated by the Pulsar program, are indicated by asterices


B
Before HX
After HX
ACTH (pmol/l)
Cortisol (nmol/l)
α-MSH (pmol/l)
GH (µg/l)
Hours of day
Hours of day

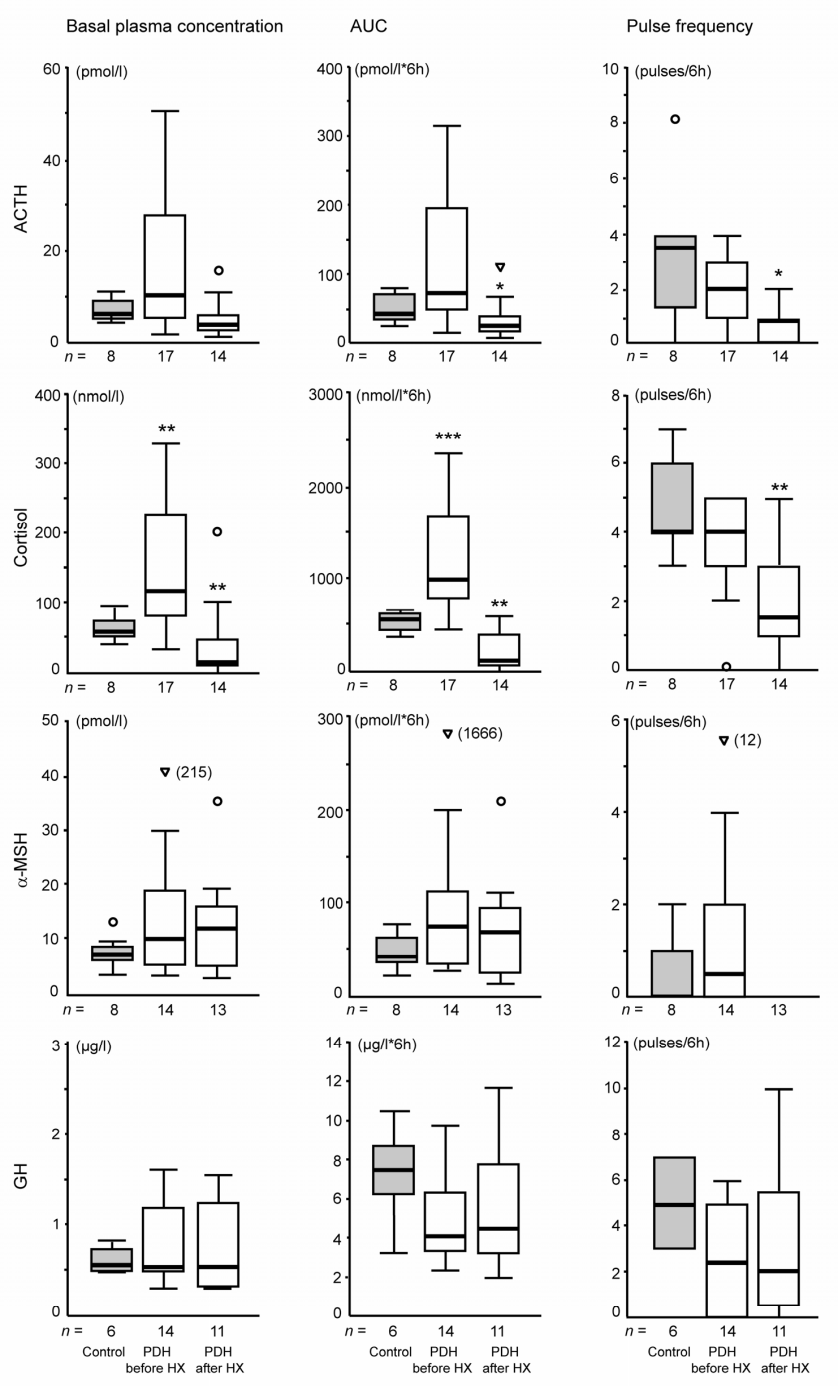

Basal plasma concentration
AUC
Pulse frequency
ACTH
Cortisol
α-MSH
GH
(pmol/l)
(pmol/l*6h)
(pulses/6h)
(nmol/l)
(nmol/l*6h)
(µg/l)
(µg/l*6h)
(215)
(1666)
(12)
n = 8 17 14
n = 8 14 13
n = 6 14 11
Control
PDH before HX
PDH after HX

Figure 2. Box-plot graphs (median, interquartile and total range) for the basal plasma hormone concentration, the area under the curve (AUC), and the pulse frequency of ACTH, cortisol, α-MSH and GH in control dogs and in dogs with pituitary-dependent hyperadrenocorticism (PDH) before and 2-4 months after hypophysectomy (HX). Blood samples were collected at 10-min intervals for 6 hours. Outliers are indicated with circles; extreme values with triangles. For values that are off scale the actual value is given within brackets. Significant differences between groups are indicated with asterices above the boxplots. Asterices above the boxes for PDH dogs before HX indicate differences compared to the control dogs and asterices above the boxes for PDH dogs after HX indicate differences compared to PDH dogs before HX; * denotes P <0.05, ** P <0.01 and *** P <0.001.

Discussion

The results of this study demonstrate that the pulsatile nature of pituitary hormone release is maintained in pituitary-dependent hyperadrenocorticism. Significant pulses of ACTH (and consequently cortisol), α-MSH, and GH were identified in the plasma profiles of dogs with PDH. Interestingly, significant pulses were also observed in some of the cases in remission after hypophysectomy. The presence of significant ACTH pulses after surgery was identified as a risk factor for recurrence of hyperadrenocorticism.

Before surgery, the pulse frequencies of ACTH and cortisol in the PDH cases were not different from those in the controls, which is in agreement with previous reports on dogs[36] and on humans with Cushing's disease.[49] In the present study, the AUC for ACTH and ACTH pulse frequency decreased after hypophysectomy. Also, the basal plasma cortisol concentration, the AUC for cortisol, and the cortisol pulse frequency decreased significantly and there was less co-occurrence between ACTH and cortisol pulses after surgery. In accordance with the relatively low values of the plasma profile characteristics for ACTH and cortisol after hypophysectomy, remission was achieved in all 16 dogs that survived the early postoperative period.

In this study, complete hypophysectomy with elimination of all ACTH-producing cells was the surgical goal. Hypophysectomy is consistent with a substantial reduction in the number of corticotropic cells which leads to remission of hyperadrenocorticism. Nevertheless, there was residual ACTH secretion after hypophysectomy. Corticotropic cells are fairly resistant to elimination and complete removal of the pituitary gland is difficult to achieve.[11] Also, in what macroscopically appears to be an empty pituitary fossa, small remnant microscopic islets of pituitary cells capable of hormone production and response to CRH stimulation test have been found after hypophysectomy in both controls and PDH dogs.[1,28,29,35] The most likely origin of these cells are remnants from an incompletely removed pars distalis adenohypophysis (which easily falls apart upon manipulation) or, in the PDH dogs, remnant adenomateous corticotropic cells. Less likely, the remnant cells may also originate from differentiated pituitary stem cells or from accessory pituitary tissue that, in the dog, is sporadically found in the dura mater lateral to the pituitary.[56]

In previous reports, no remnant pituitary cells were identified on the ventral hypothalamic diencephalons in hypophysectomized experimental animals.[1,28,29] Therefore, it can be assumed that the pituitary-hypothalamic portal system is completely impeded and that the residual hormone secretion activity measured in this study originates from the microscopic islets in the pituitary fossa. Interestingly, the residual ACTH secretion was pulsatile in character. It is possible this could be due to the influence of CRH, to which the corticotropic

cells in the pituitary residual islets are able to respond,[28,29] reaching the pituitary cells on an alternative vascular route or through the cerebrospinal fluid from the opened third ventricle after hypophysectomy. The residual pulsatile ACTH secretion after hypophysectomy may also be explained by intrinsic ACTH pulsatility,[10] by release of vasopressin from the hypothalamus or by CRH independent secretory capacity of corticotropic cells.[8]

In agreement with earlier observations,[19,36] α-MSH was secreted in a pulsatile fashion in both the controls and the PDH dogs. Under basal conditions the pars intermedia of the canine adenohypophysis is under a strong and almost permanent dopaminergic inhibition.[17,29,36,38] The significant α-MSH pulses are probably the result of a temporary decrease in the tonic hypothalamic dopaminergic inhibitory control. Administration of a dopamine antagonist such as haloperidol results in a significant release of α-MSH.[19,36]

The α-MSH pulsatility was similar in the PDH dogs and the controls. After hypophysectomy there were no α-MSH pulses. This is compatible with complete removal of the neurointermediate lobe. In a previous study on pituitary function after hypophysectomy in experimental animals, administration of the dopamine-antagonist haloperidol caused no elevation of the basal plasma α-MSH concentration, whereas before surgery there was a 30-fold increase.[29]

Several α-MSH pulses co-occurred with ACTH pulses which is explained by co-release of ACTH and α-MSH from the pars intermedia[19] by the same secretagogue or stimulatory event. Previous studies demonstrated that the dopamine-antagonist haloperidol increases the plasma concentrations of both α-MSH and ACTH.[17,19] The haloperidol-stimulated secretion of α-MSH reached its maximum value within 10 min, whereas the haloperidol-stimulated secretion of ACTH reached its maximum value after 60 min. Therefore, an α-MSH pulse preceded an ACTH pulse in some cases in the present study.

Dogs with PDH and humans with Cushing's disease secret less GH in pulses than healthy individuals.[26,55] The same was observed in the present study, although no statistical significance was reached. Sustained exposure to supraphysiological amounts of glucocorticoids inhibits pulsatile GH secretion and blunts the GH response to stimuli mainly by altering the hypothalamic somatostatin tone.[34]

In the group of PDH dogs, hypophysectomy caused no further significant decrease of the already low plasma GH concentrations. However, in some individual cases the decline of the basal GH values was distinct. These findings are in agreement with a previous study in which hypophysectomy led to a significant decrease of the plasma GH concentrations[28]. Plasma GH concentrations unaffected by hypophysectomy is most likely the result of secretion by residual somatotropic cells in the pituitary fossa.[15,28,29] Another explanation is extra-pituitary GH production that occurs in many tissues under both normal and pathologic conditions.[13,14,25,33,39,46,53] This extra-pituitary GH is mainly thought to have local autocrine and paracrine effects, but it may also reach the systemic circulation. For example, progesterone-induced GH release from the mammary gland during the luteal phase may increase the plasma GH concentration in bitches.[20] However, hypophysectomy removes the FSH- and LH-secreting gonadotropes (resulting in permanent anoestrus and a very low plasma progesterone concentration), and therefore it is unlikely that circulating GH after hypophysectomy is of mammary origin.

There was no association between preoperative characteristics of the plasma hormone profiles and recurrence of hyperadrenocorticism, which is in agreement with the results of a study after transsphenoidal hypophysectomy in humans with Cushing's disease.[5] The number

of significant ACTH pulses after hypophysectomy, however, was identified as a risk for recurrence of hyperadrenocorticism. Consequently, pulsatile ACTH secretion at eight weeks after hypophysectomy is more likely to reflect the presence of residual adenomatous than unaffected corticotropic cells in the pituitary fossa. In a previous study, CRH stimulation 8 weeks after hypophysectomy effectively identified dogs with residual disease but failed to differentiate between the cases that developed recurrence of hyperadrenocorticism and those that remained in remission.[28] Measuring the plasma profiles of ACTH gives additional information on the long-term prognosis. The slow growth rate of the corticotropic adenoma may explain why hyperadrenocorticism recurs in 1.5 to 5 years. There was also a tendency that high postoperative AUC for GH was associated with a low risk of recurrence, which may be a reflection of normalized GH secretion of the residual somatotropic cells after successful elimination of inhibition by high cortisol levels.

In conclusion, the results of the present study indicate that in PDH dogs ACTH, cortisol, α-MSH, and GH are secreted in a pulsatile fashion, and that hypophysectomy effectively reduces the secretion of ACTH and cortisol. The presence of ACTH pulses after hypophysectomy is associated with a higher risk of recurrence of hyperadrenocorticism.

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CHAPTER 6

Peri-operative plasma profile of adrenocorticotrophic hormone predicts recurrence after transsphenoidal hypophysectomy for the treatment of pituitary-dependent hyperadrenocorticism in dogs

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Abstract

Transsphenoidal hypophysectomy is an effective treatment for pituitary-dependent hyperadrenocorticism (PDH) in the dog. However, recurrences occur in about 25% of the cases after initial remission. The aim of this study was to analyze the predictive value of immediate postoperative plasma concentrations of adrenocorticotropic stimulating hormone (ACTH), α-melanocyte stimulating hormone (α–MSH), growth hormone (GH) and cortisol for recurrence after transsphenoidal hypophysectomy in dogs with PDH.

Transsphenoidal hypophysectomy was performed in 55 dogs with PDH. Plasma hormone concentrations were measured the day before surgery and 1, 2, 3, 4, 5 and 24 to 48 hours after the removal of the pituitary gland. Absolute values and the degree of decrease were analyzed for their prognostic value for recurrence.

Dogs with persistent disease were readily identified with the perioperative profile. Univariate Cox's proportional-hazard analysis revealed that postoperative plasma ACTH, cortisol and pre-and postoperative α-MSH concentration are prognostic for surgical outcome.

It can be concluded that perioperative measurement of plasma concentrations of ACTH is easily performed and a valuable analysis for early postoperative evaluation of dogs with PDH after transsphenoidal hypophysectomy.

Despite high initial remission rates, long-term recurrence is a well recognized complication after transsphenoidal hypophysectomy in dogs with pituitary-dependent hyperadrenocorticism (PDH)[20,27] as well as after transsphenoidal pituitary surgery in humans with Cushing's disease.[2,5,8,13,21,32,33,37] Therefore, much effort has been put into the search of a good predictor of recurrence and a reliable tool for the evaluation of completeness of tumor removal. For example, it has been shown in both species that recurrences are more common in patients with enlarged pituitaries.[4,19,20,46] In addition, we recently showed that dogs with pituitary enlargement, high pre-operative plasma concentrations of α-melanocyte stimulating hormone (α-MSH), high urinary cortisol-to-creatinine ratio (UCCR) and increased thickness of the sphenoid bone had an increased risk of recurrence.[19] In human patients, young age, presence of major depression and high pre-treatment urinary cortisol levels are associated with recurrences.[5,44]

For postoperative evaluation in the dog, the UCCR has been used to define residual disease, remission and recurrence. UCCR values in the upper normal range at 6-8 weeks after surgery are associated with a higher frequency of recurrences at long-term follow-up.[19] For postoperative evaluation of pituitary surgery in humans, the plasma cortisol level or urinary cortisol excretion is commonly used. There are different protocols including evaluation during the first week after surgery, with or without postoperative cortison medication[2,8,15,37,41,43,47,53] or evaluation of urinary cortisol excretion 3 month after.[35]

In addition, there are protocols for intraoperative[11,25] and postoperative measurements of plasma ACTH concentrations, perioperative measurements of plasma cortisol concentrations,[26] as well as stimulations tests with corticotropin-releasing hormone (CRH),[3,22,42] desmopressin,[9,10,22,24,48] and metyrapone.[49] However, a test that is predictive for the individual patient remains to be developed.[1]

In dogs with PDH, a CRH test performed at 8 weeks after hypophysectomy, failed to identify the dogs in which the disease would recur. In the majority of 35 dogs in which initial remission of hyperadrenocorticism was achieved, there were still small significant residual plasma ACTH and cortisol reponses to CRH at 8 weeks after hypophysectomy.[28] Three of these 35 dogs developed a recurrence at 7 to 16 months after surgery. Similar small residual ACTH and cortisol responses were also seen in normal dogs after hypophysectomy.[29] In dogs with PDH, identifiable postoperative pulsatile elevations of plasma ACTH concentrations, 8 weeks after hypophysectomy, were associated with an increased risk of recurrence.[18] The aim of the present study was to evaluate the predictive value of the early, immediate postoperative plasma ACTH-, cortisol, α-MSH, and GH concentrations for recurrences of hyperadrenocorticism after transsphenoidal hypophysectomy in dogs with PDH.

Materials and Methods

Animals

Fifty-five dogs with PDH, referred to the Department of Clinical Sciences of Companion Animals, Utrecht University, over a 4.5-year period, underwent transsphenoidal hypophysectomy as primary treatment for PDH. All dogs were operated by the same veterinary neurosurgeon. Dogs of 30 different breeds and cross-bred dogs were included. There were 1 Alaskan Malamute, 1 American Staffordhshire Terrier, 1 Beagle, 2 Bearded Collies, 1 Belgian Shepherd Dog, 1 Bolognezer, 1 Bouvier des Flandres, 3 Boxers, 2 Dachshunds,

1 Dog Argentino, 2 German Pointers, 1 English Cocker Spaniel, 1 Fox Terrier, 1 French Bulldog, 1 Irish Terrier, 2 Jack Russel Terriers, 4 Labrador Retrievers, 4 Malteses, 1 Medium-sized Poodle, 1 Rottweiler, 1 Shipperke, 1 Scottish Collie, 1 Scottish Terrier, 1 Shar Pei, 1 Stabyhound, 1 Welsh Terrier, 1 West Highland White Terrier, 3 Yorkshire Terriers and 9 cross-bred dogs. There were 22 male dogs (10 castrated) and 29 female dogs (21 castrated). The age at time of surgery ranged from 4 to 13 years (median, 8.4 years). The body-weight ranged from 4 to 61 kg (median, 19 kg).

Diagnosis

The diagnosis of hyperadrenocorticism was based upon the averaged UCCR in two consecutive morning urine samples combined with a high-dose dexamethasone suppression test, as described earlier.[40,45] After collection of the second urine sample, three oral doses of 0.1 mg dexamethasone per kg body weight were administered at 8-h intervals and the next morning a third urine sample was collected. When the UCCR in the third sample was less than 50% of the mean in the first 2 samples, the dog was categorized as being responsive to dexamethasone suppression and PDH was diagnosed.[16] In cases with less than 50% suppression of the UCCR in the third sample, dexamethasone-resistent PDH was demonstrated by measurements of plasma ACTH concentrations and further supported by visualization of the adrenals by ultrasonography and pituitary imaging.[6,39,51,52]

Surgery

Transsphenoidal hypophysectomy was performed according to a microsurgical technique described previously.[31] Following removal of the pituitary gland, treatment was started 1 drop of 0.01% desmopressin (Minirin; Ferring, Hoofddorp, The Netherlands) and continued every 8 h in the conjunctival sac. Intravenous administration of 1 mg hydrocortisone (Solu-cortef; Upjohn, Ede, The Netherlands) per kg body weight was started 5 hour after removal of the pituitary gland (after the last blood sample for hormone analysis) and continued every 6 h until the dog resumed drinking and eating. Further postoperative treatment was according to the same protocol as described previously.[30] The dogs were kept on life-long oral 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) 15 µg/kg, every 12 hours. Desmopressin was administered for 2 weeks routinely and continued if polyuria due to central diabetes insipidus persisted.[30] The dogs were re-examined after 8 weeks. After surgery, UCCR was measured (in duplicate) 24 h after cortisone acetate medication at 2 weeks, 8 weeks, 6 months and thereafter once a year, or more frequently in cases with suspected recurrence. Follow-up reports were obtained from the routine follow-up examinations in the hospital and during telephone conversations with the owner and/or the referring veterinarian. Postoperative mortality was defined as death within 4 weeks after surgery irrespective of the cause of death. Residual disease was defined as early postoperative (< 2 months after surgery) UCCR $\geq 10 \times 10^{-6}$ and no resolution of clinical signs and/or remnant pituitary tumor tissue on early postoperative CT or MRI scans. Remission was defined as UCCR $< 10 \times 10^{-6}$ and resolution of clinical signs of hyperadrenocorticism. Recurrence was defined as UCCR $\geq 10 \times 10^{-6}$ and return of signs and symptoms of hyperadrenocorticism after initial complete remission.

Blood sampling

Two blood samples were colleted on the day before surgery (-24 h). After surgery, the first blood sample (1) was collected when the dog was still on the operating table, approximately 1 h after extraction of the pituitary gland. The following samples were collected at 2, 3, 4, and 5 h after surgery. Hydrocortisone medication was only startetd after the 5 h sample. The last blood sample was collected the following day (24 h) or the day after (48 h), at 9 am before medication of the dog. Blood samples were collected in pre-chilled EDTA tubes and kept on ice and centrifuged at 4 ºC, for 10-12 minutes. The plasma was transferred and divided into 3 (4 when GH was included) polypropylene tubes, and stored at -20 ºC until analyzed.

Hormone determinations

Plasma ACTH concentration was measured by a commercially available two-site immuno-radiometric assay (IRMA) (Nichols Institute, Wijchen, The Netherlands). The antiserum was highly specific for ACTH (1-39). A polyclonal antibody was used that specifically binds the C-terminal region of ACTH. The radioiodinated monoclonal antibody bound only to the N-terminal region of ACTH. The intra-assay CV was 3.2%, the inter-assay coefficient of variation (CV) was 7.8%, and the sensitivity was 0.22 pmol/l. There was no cross-reaction between the antiserum and α-MSH or ACTH precursors.[6,36]

Plasma cortisol concentration was measured by a solid phase ^{125}I radio-immuno assay (RIA) (Coat-A-Count® Cortisol, Diagnostic Products Corporation, Los Angeles, USA). The antiserum was highly specific for cortisol, with very low cross-reactivity to other compounds that were present in patient samples. Protein, lipids, bilirubin or hemolysis had no significant effect on the assay. The intra-assay CV was 4%, the inter-assay CV was 4.5-6.3%, and the sensitivity was 5.5 nmol/l**.**

Plasma concentration of α-MSH was measured by RIA without extraction according to methods described previously [38]. The intra-assay CV was 10%, the inter-assay CV was 23%, and the sensitivity was 3 pmol/l. The antiserum had less than 0.1% cross-reactivity with ACTH (1-39) and 4% cross-reactivity with ACTH (1-24). Plasma α-MSH concentrations equal to or more than 36 pmol/l were considered increased.[6]

Plasma growth hormone (GH) concentration was measured by a homologous RIA as described previously by Eigenmann & Eigenmann.[14] The intra-assay CV was 3.8%, the inter-assay CV was 7.2%, and the sensitivity was 0.3 µg/l. The degree of cross-reaction of canine prolactin was 2%.[14]

Cox's proportional-hazard analysis

Analyses were performed with SPSS statistical package (SPSS Benelux BV, Gorinchem, The Netherlands). The disease-free interval was calculated for dogs in which remission of hyperadrenocorticism was obtained, and was defined as the interval between the date of surgery and the date on which the dog was last known to be free of signs of hyperadrenocorticism and to have UCCR $< 10 \times 10^{-6}$, or the date of recurrence of signs of hyperadrenocorticism accompanied by UCCR $\geq 10 \times 10^{-6}$. Dogs that had died from non-related causes and dogs that were still alive at the time of follow-up were counted as censored cases. Variables were first analyzed with univariate Cox's proportional-hazard analysis. The variables that were entered into the analysis were absolute plasma concentrations of ACTH, cortisol, α-MSH and GH on the day before surgery (-24h), and at 1, 2, 3, 4, 5, and

24-48 hours after removal of the pituitary gland. The -24 h value was the mean of two samples withdrawn with an interval of 10-15 minutes. Also, each postoperative value was normalized by the -24 h value, and entered the equation. The remaining proportion of plasma hormone concentrations normalized by the preoperative starting value was calculated for each postoperative value. The variable with the lowest P value for each hormone was entered into a multivariate Cox's proportional-hazard analysis (Forward Stepwise (Contitional LR)).

Results

Persistent disease

Three dogs with normal sized pituitaries were classified as having persistent disease based on the 8-week postoperative UCCR. A complete hypophysectomy was reported in the surgery report for all three dogs and a pituitary adenoma was verified in 2 cases and suspected in one case. Dog 1 had persistent high plasma cortisol levels despite low plasma ACTH levels. The profile of dog 2 and 3 showed persistent ACTH secretion (Figure 1).

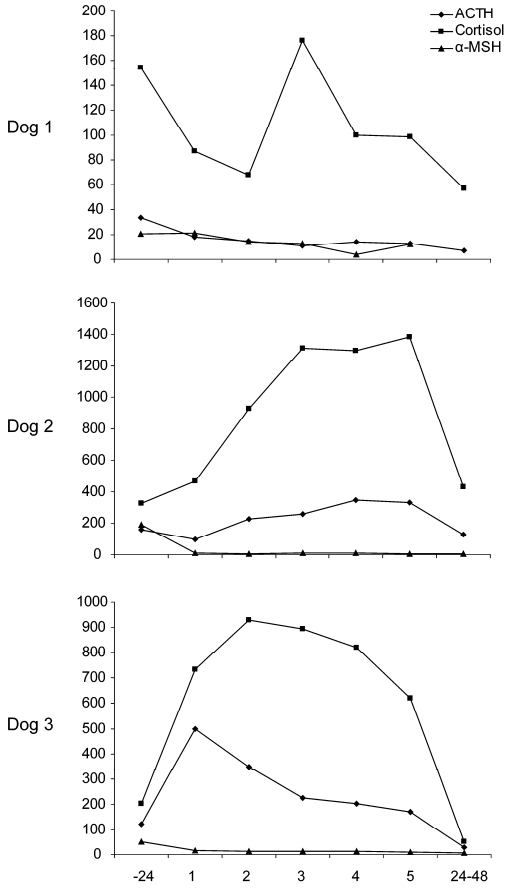

Figure 1. Perioperative plasma hormone profiles after transsphenoidal hypophysectomy for the treatment of pituitary-dependent hyperadrenocorticism in 3 dogs with residual disease after surgery. Blood samples were taken on the day before surgery (-24) and at 1, 2, 3, 4, 5 and 24-48 hours after removal of the pituitary gland.

Remission and Recurrence

Fourty-eight dogs went into remission (Figure 2). Median disease-free period was 569 days (range, 55-1927). Hyperadrenocorticism recurred in 12 dogs (25%) after median 255 days (range, 54 to 1281 days). The plasma profiles of these dogs are shown in Figure 3.

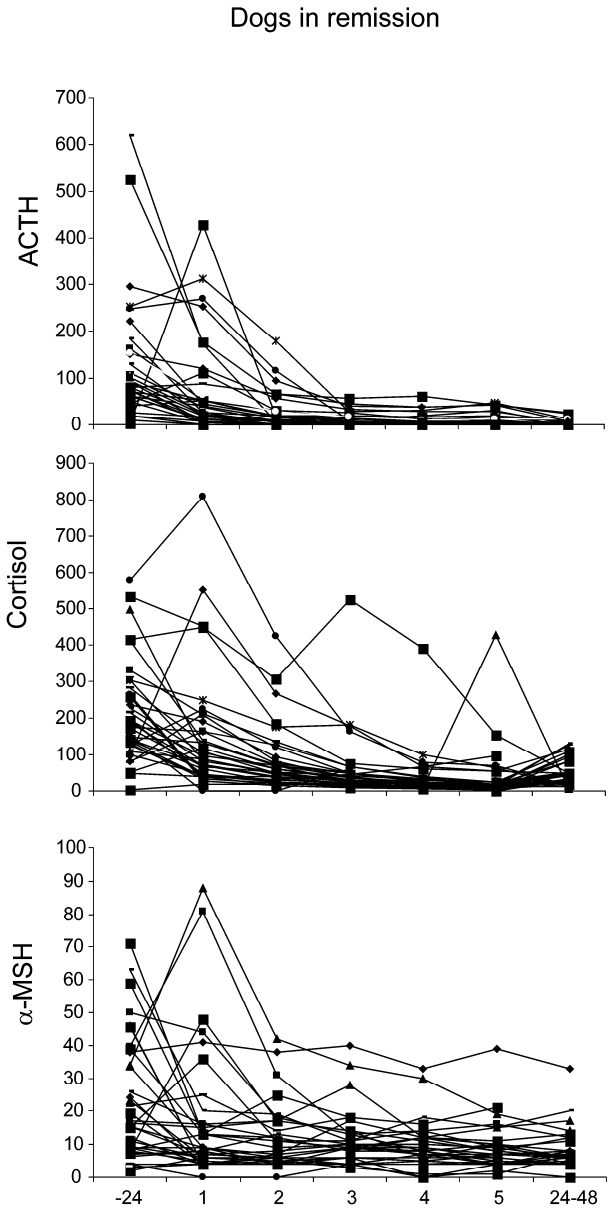

Figure 2. Perioperative plasma hormone profiles after transsphenoidal hypophysectomy for the treatment of pituitary-dependent hyperadrenocorticism in 36 dogs that remained in remission until time of evaluation. Blood samples were taken on the day before surgery (-24) and at 1, 2, 3, 4, 5 and 24-48 hours after removal of the pituitary gland.

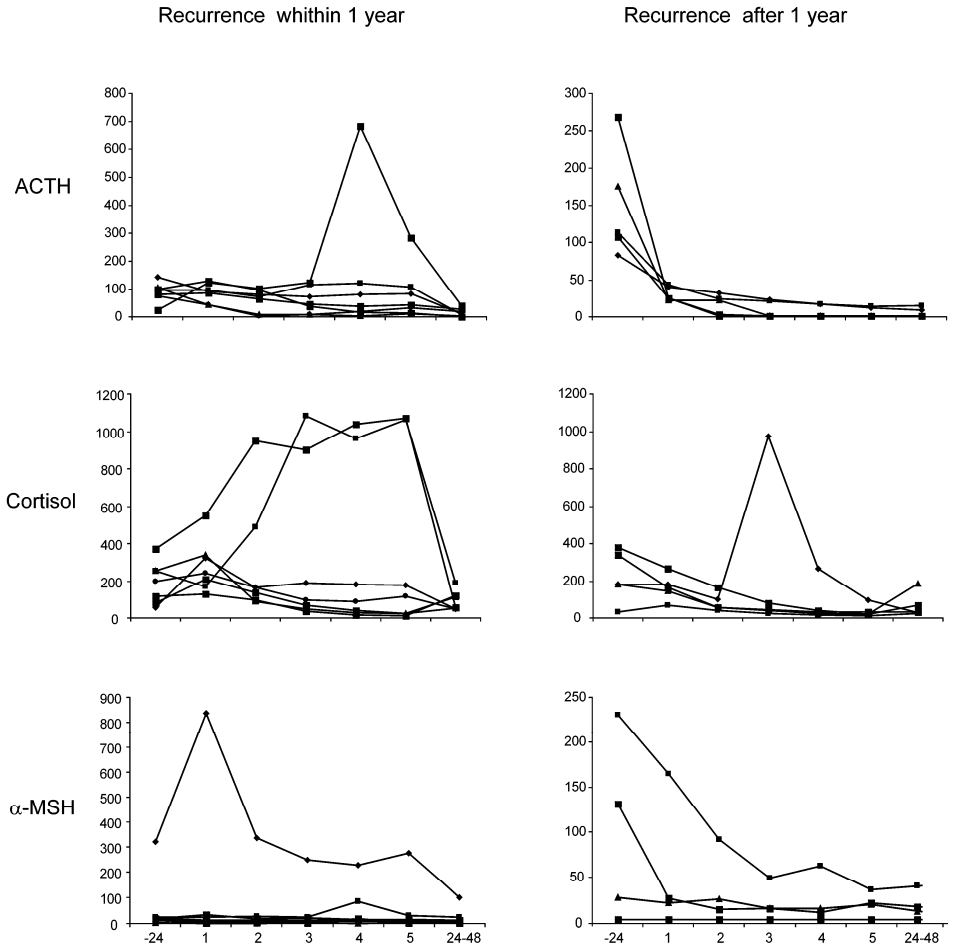

Figure 3. Perioperative plasma hormone profiles after transsphenoidal hypophysectomy for the treatment of pituitary-dependent hyperadrenocorticism in 12 dogs that developed recurrence of the disease after a period of initial remission. ACTH: adrenocorticotropic hormone; α-MSH: α-melanocyte-stimulating hormone. Blood samples were taken on the day before surgery (-24) and at 1, 2, 3, 4, 5 and 24-48 hours after removal of the pituitary gland.

Cox's proportional-hazard analysis

The ACTH-values at 3, 4, 5, and 24-48 hours after removal of the pituitary gland were significant for predicting recurrences. The plasma ACTH concentrations at 3 hours after removal of the pituitary gland had the smallest P-value and highest hazard ratio (HR) (Table 1). A high proportion of plasma ACTH concentration at 4 and 5 hour after removal of the pituitary gland was inversely associated with an increased risk for recurrence (Table 2). Plasma concentration of α-MSH was predictive for recurrence independent of time point when the sample was collected (Table 1). A high proportion of plasma α-MSH concentration

at 4 hours after removal of the pituitary gland was associated with an increased risk for recurrence (Table 2). All postoperative measurements of cortisol were significant for disease-free period (Table 1). A high normalized plasma cortisol concentration 5 hour after removal of the pituitary gland was associated with an increased risk for recurrence (Table 2). No measurement of GH, before or after surgery, was predictive for recurrence (Table 1 and 2).

Table 1

Univariate Cox's proportional-hazard analysis of absolute plasma hormone concentration for disease-free periods after transsphenoidal hypophysectomy for the treatment of pituitary-dependent hyperadrenocorticism in dogs.

Variable	*n* events	P	HR	95% CI
ACTH -24	12	0.828	0.999	0.994-1.004
ACTH 1	12	0.911	1.000	0.993-1.006
ACTH 2	12	0.085	1.011	0.999-1.023
ACTH 3	12	0.000	1.033	1.016-1.051
ACTH 4	12	0.003	1.006	1.002-1.011
ACTH 5	12	0.001	1.019	1.007-1.030
ACTH 24-48	12	0.000	1.106	1.048-1.167
α-MSH -24	12	0.023	1.007	1.001-1.013
α-MSH 1	12	0.044	1.003	1.000-1.005
α-MSH 2	12	0.028	1.007	1.001-1.013
α-MSH 3	12	0.030	1.009	1.001-1.018
α-MSH 4	12	0.007	1.011	1.003-1.020
α-MSH 5	12	0.029	1.008	1.001-1.016
α-MSH 24-48	12	0.011	1.026	1.006-1.046
Cortisol -24	12	0.746	0.999	0.995-1.004
Cortisol 1	12	0.050	1.002	1.000-1.005
Cortisol 2	12	0.001	1.005	1.002-1.008
Cortisol 3	12	0.006	1.002	1.001-1.003
Cortisol 4	12	0.001	1.004	1.002-1.006
Cortisol 5	12	0.001	1.003	1.001-1.005
Cortisol 24-48	12	0.025	1.013	1.002-1.024
GH -24	6	0.424	0.439	0.058-3.301
GH 1	6	0.180	1.851	0.752-4.553
GH 2	6	0.954	0.967	0.310-3.019
GH 3	6	0.862	1.184	0.176-7.957
GH 4	6	0.108	5.115	0.701-37.34
GH 5	6	0.697	1.591	0.154-16.47
GH 24-48	6	0.357	0.003	0.000-737.2

n = number; ACTH = adrenocorticotropic hormone; α-MSH = α-melanocyte-stimulating hormone; GH = growth hormone; P = probability; HR = hazard ratio; CI = confidence interval

Table 2
Univariate Cox's proportional-hazard analysis of plasma hormone concentrations normalized (nor) by preoperative starting values for disease-free periods after transsphenoidal hypophysectomy for the treatment of pituitary-dependent hyperadrenocorticism in dogs.

Variable	*n* events	P	HR	95% CI
ACTH 1 ^{nor}	12	0.676	0.985	0.918-1.057
ACTH 2 ^{nor}	12	0.345	1.196	0.824-1.736
ACTH 3 ^{nor}	12	0.258	1.200	0.875-1.645
ACTH 4 ^{nor}	12	0.008	1.149	1.037-1.273
ACTH 5 ^{nor}	12	0.016	1.319	1.053-1.653
ACTH 24-48 ^{nor}	12	0.140	2.324	0.759-7.119
α-MSH 1 ^{nor}	12	0.842	0.943	0.529-1.679
α-MSH 2 ^{nor}	12	0.922	1.051	0.394-2.803
α-MSH 3 ^{nor}	12	0.903	0.930	0.291-2.977
α-MSH 4 ^{nor}	12	0.017	2.379	1.166-4.856
α-MSH 5 ^{nor}	12	0.234	2.518	0.550-11.514
α-MSH 24-48 ^{nor}	12	0.978	1.012	0.429-2.388
Cortisol 1 ^{nor}	12	0.142	1.221	0.935-1.596
Cortisol 2 ^{nor}	12	0.180	1.212	0.915-1.605
Cortisol 3 ^{nor}	12	0.108	1.206	0.960-1.516
Cortisol 4 ^{nor}	12	0.058	1.348	0.9901834
Cortisol 5 ^{nor}	12	0.015	1.703	1.111-2.610
Cortisol 24-48 ^{nor}	12	0.684	0.958	0.778-1.179
GH 1 ^{nor}	6	0.055	2.023	0.985-4.156
GH 2 ^{nor}	6	0.618	1.446	0.339-6.163
GH 3 ^{nor}	6	0.170	2.357	0.692-8.026
GH 4 ^{nor}	6	0.108	4.293	0.725-25.406
GH 5 ^{nor}	6	0.390	1.712	0.503-5.830
GH 24-48 ^{nor}	6	0.512	0.453	0.043-4.821

ACTH = adrenocorticotropic hormone, per = percentage of value before surgery; α-MSH = α-melanocyte-stimulating hormone; GH = growth hormone; P = probability; HR = hazard ratio; CI = confidence interval

Table 3
Multivariate Cox's proportional-hazard analysis (Forward stepwise (Conditional LR)) for disease-free periods after transsphenoidal hypophysectomy for the treatment of pituitary–dependent hyperadrenocorticism in dogs

Variables entered	*n* events	Significant variables	P	HR	95% CI
ACTH 24-48 α-MSH 4 Cortisol 5	12	ACTH 24-48 α-MSH	0.001 0.041	1.100 1.009	1.038-1.164 1.000-1.018
ACTH 4 ^{nor} α -MSH 4 ^{nor} Cortisol 5 ^{nor}	12	ACTH 4 ^{nor}	0.008	1.148	1.036-1.271

n: number; ACTH: adrenocorticotropic hormone, per: percentage of value before surgery; α-MSH: α-melanocyte-stimulating hormone; P: probability; HR: hazard ratio; CI: confidence interval; nor: normalized

Discussion

In the present study we show that the measurement of perioperative plasma concentrations of ACTH, α-MSH and cortisol gives useful information for the evaluation of surgical outcome after transsphenoidal hypophysectomy for the treatment of PDH in dogs.

For the dog the aim of pituitary surgery is hypophysectomy whereas in humans it is selective adenomectomy. Therefore, in dogs very low ACTH values after hypophysectomy reflect complete absence of the pituitary gland (including the adenoma) whereas in humans very low ACTH values after selective adenomectomy reflect long-term suppression of normal pituitary corticotrophs.

Both the absolute value and the proportion plasma hormone concentration compared to the preoperative value were analyzed. The plasma half-life of ACTH and approximately 20 minutes in dogs with PDH[17,34] which is similar to what has been reported in humans.[50] This implicates that the plasma hormone concentrations 3 hours after the removal of the pituitary gland including the adenoma would approach the zero value, also in those dogs with the highest preoperative plasma ACTH values, but may be somewhat slower if the second, slow elimination phase has been entered.[34] Indeed this observation is supported by a study in humans with Cushing's disease where the venous ACTH concentration was measured intraoperatively and after transsphenoidal selective adenomectomy. Already 2 hours after surgery, a considerable decline in ACTH was observed which was even lower on the day after surgery.[11] In this report blood was collected until 2 hours directly after surgery, and on the day after surgery. Unfortunately, sampling was not continued after 2 hours, thus it is not known when the plasma concentrations reaches the low steady level of plasma concentrations.

In agreement with our previous findings, high absolute plasma α-MSH concentration before surgery was associated with an increased risk of recurrence.[19] This may be ascribed to more aggressive characteristics of these corticotroph tumors with a likely origin from the pars intermedia. The postoperative profile contributes with additional information about the completeness of the removal of the pituitary gland including the adenoma.

Plasma cortisol has a longer half-life (in humans approximately 50 minutes,[23] and the decrease of plasma cortisol concentations is expected to be less steep than that of ACTH. The plasma cortisol concentration is also dependent on the activity of the adrenal glands. Luedecke and co-workers (1996) described different patterns of decline of plasma cortisol concentrations after selective removal of pituitary microadenomas before starting substitution therapy. Patients with long-term cure may show both a rapid and a slow decline, both ending at low 24-hour postoperative cortisol levels.[26]

In our study, the early postoperative ACTH values were most informative for surgical outcome, however, the plasma α-MSH and cortisol values gave additional information. The hourly-repeated measurement has the advantage giving a trend of the changes and identifying peaks in the plasma concentrations, compared to single measurements.

The protocol presented in this study includes, at time of tumor removal, the administration of desmopressin at an approximate dose of 4 µg in the conjunctival sac, 3 times daily starting immediately after surgery. Desmopressin is a long-acting (8 h) vasopressin analogue that stimulates ACTH secretion. Therefore, administration of desmopressin immediately after surgery, may be regarded as a low-dose desmopressin stimulation test. A postoperative desmopressin-stimulation test has been used previously to study pituitary tumor removal after surgery.[48] In this study, performed 3 to 6 months postoperatively, 3 out of 5 patients with

recurrences were identified. Also, there are indications that a paradoxical reaction on desmopressin-stimulation (rise in ACTH without change in plasma cortisol concentration) may proceed a recurrence.[12] Such information may become valuable in the future if medical treatment addressing the function of the pituitary cells are being developed.[7]

The postoperataive decline in ACTH is informative for cases with residual disease and early recurrences. Also in this study it was shown, that low postoperative ACTH values cannot identify every recurrence. Re-growth of tumor tissue is the most likely cause. The time course for this is dependent on the amount of cells that are left behind and their ability to re-establish function and proliferation. In these cases one may use the additional information available such as pituitary enlargement, high plasma concentrations of α-MSH, thickness of the sphenoid bone, and high preoperative UCCR that are risk factors for recurrence.[19] We can therefore conclude that a close follow-up is mandatory, also on the long term, of the dogs with PDH after transsphenoidal hypophysectomy.

It can be concluded that combined perioperative measurement of ACTH, cortisol and α-MSH contributes valuable information for the evaluation of the patient after transsphenoidal hypophysectomy for the treatment of PDH in dogs. A close follow-up of the patients also on the long-term is nevertheless mandatory.

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Oncogenesis of corticotroph pituitary adenomas in dogs

CHAPTER 7

Expression and mutation analysis of Tpit in the canine pituitary gland and corticotroph adenomas

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Abstract

Pituitary-dependent hyperadrenocorticism (PDH) in dogs is caused by a pituitary corticotroph adenoma. Although PDH is a common disorder in dogs, little is known about the underlying pathogenesis. In the pituitary glands of humans and mice, the pro-opiomelanocortin (POMC)-expressing cell lineages, the corticotrophs and melanotrophs, have a specific marker in common, the T-box transcription factor Tpit (Tbx19), which is obligate for POMC expression. Tpit also regulates the late differentiation of the corticotrophs and melanotrophs, and therefore may contribute to the pathogenesis of the corticotroph adenomas. The aim of this study was to perform an expression and mutation analysis of Tpit in the normal canine pituitary and in corticotroph adenomas. The distribution of the Tpit protein in the pituitary gland was studied with immunohistochemistry and the expression of the gene with RT-PCR. The coding region of Tpit cDNA from 14 dogs with PDH was screened for mutations. Tpit was expressed in corticotroph and melanotroph cells of the normal and adenomatous canine pituitary, and remained present in non-adenomatous corticotrophs of pituitaries from PDH dogs. No tumor-specific mutation in the Tpit cDNA from the corticotroph adenomas was found. However, a missense polymorphism in the highly conserved DNA-binding domain, the T-box, was discovered in one dog.

It is concluded that Tpit can be used as a reliable marker for the corticotroph and melanotroph cells in the canine pituitary tissue and that mutations in the Tpit gene are unlikely to play a major role in the pathogenesis of canine corticotroph adenomas.

Pituitary-dependent hyperadrenocorticism (PDH) is a common endocrinopathy in the dog, that is caused by an adrenocorticotropic hormone (ACTH) secreting corticotroph adenoma.[17] Although 60 years has passed since the disease was first described in the dog, the underlying pathogenesis remains unknown.[5] There are only a few reports on the molecular characteristics.[25] The genetic mechanisms of oncogenesis may be related to dysregulation of factors involved in normal differentiation processes of the tumorous tissue.[4] Members of the T-box family of transcription factors have a highly conserved DNA-binding domain in common,[11] the T-box, and are required for both early cell-fate decision, differentiation and organogenesis of the pituitary gland and other tissues, and reported to be involved in carcinogenesis.[19,26,27] In the pituitary gland, the expression of the *Brachyury* (T) related pituitary T-box factor Tpit (Tbx19) is a specific marker for the pro-opiomelanocortin (POMC) expressing cell lineages; the ACTH producing corticotrophs and the α-melanocyte-stimulating hormone producing melanotrophs.[9,21] Among the hormone producing cell lineages of the pituitary gland, the corticotrophs are the first to reach terminal differentiation.[7,20] Tpit has an important role in the late phase of this process,[9,16] and is important for the maintenance of both corticotroph and melanotroph cells.[16]

Tpit acts as an obligate transcription factor for POMC expression.[9,15] Tpit binds to the POMC-promoter and interacts with the corticotroph up-stream transcription element in co-operation with the homeoprotein Pitx1[9] to activate POMC-transcription. Tpit recruits steroid receptor coactivator 2 (SRC-2), and is responsive to signals elicited by hypothalamic corticotrophin-releasing hormone.[10] This transcriptional activity of the Tpit/Ptx1 element is repressed by bone morphogenic protein 4, the effect of which is mediated by activated mothers against decapentaplegic homolog 1 (Smad1).[14]

The aim of the present study was to investigate the role of Tpit in the normal canine pituitary gland and in corticotroph adenomas. It was hypothesized that Tpit gain-of-function mutations, may be involved in the pathogenesis of corticotroph tumors in the dog. The Tpit protein was detected immunohistologically, the coding part of Tpit mRNA was sequenced, and 14 adenomatous pituitary glands were screened for mutations.

Materials and Methods

Animals

Pituitary tissue specimens from 23 dogs with PDH were collected at the time of transsphenoidal hypophysectomy[12] for the treatment of PDH. The study group included 1 Beagle, 1 Bearded Collie, 1 Bernese Mountain Dog, 1 Boxer, 1 Dachshund, 1 Dogo Argentino, 1 Dutch Shepherd, 3 English Cocker Spaniels, 1 Golden Retriever, 1 Havanese, 1 Hovawart, 1 Jack Russel Terrier, 1 Labrador Retriever, 1 Maltese, 1 Minature Poodle, 1 Soft Coated Retriever, 1 Vizsla, 1 Welsh Springer Spaniel, and 3 cross bred dogs. There were 12 female dogs (6 spayed), 11 males (3 castrated), median age was 8 years (range, 3-12), and median body weight was 22 kg (range, 4-59 kg).

The diagnosis of hyperadrenocorticism was based upon urinary corticoid-to-creatinine ratios (UCCR) in two consecutive morning urine samples.[24] After collection of the second urine sample, three oral doses of 0.1 mg dexamethasone per kg body weight were administered at 8-h intervals. PDH was diagnosed when the UCCR in the third sample was less than 50% of the mean of the first two samples.[6] In cases with less than 50% suppression of the

third UCCR, pituitary dependency was secured by measurements of plasma ACTH concentrations and further supported by visualization of the pituitary gland with computed tomography (CT).[2,18,22,23] The diagnosis of PDH was determined without measurement of UCCR in 3 dogs. In these dogs the diagnosis was supported by pituitary imaging and confirmed by histopathology. Twenty dogs had enlarged pituitaries based on the pituitary height-to-brain area ratio (P/B ratio > 0.31 x 10^{-2} mm^{-1}) as measured on CT images and described previously.[8] All dogs went into initial remission after surgery.

The pituitary gland of 3 healthy Labrador retrievers (2 females, 1 male, 1.5 to 3 years old) was collected directly (within 5 minutes) after euthanasia. These dogs were euthanized in other experiments which have been approved by the Ethical Committee of the Faculty of Veterinary Medicine, Utrecht University, The Netherlands.

For analysis of Tpit missense polymorphism in one Bernese mountain dog, genomic DNA isolated from blood[13] was analyzed and compared with that of 24 healthy Bernese mountain dogs. Blood sampling was performed with informed consent of the owners.

Tissue

For immunoreactive studies, pituitary tissue specimen from control and pituitary adenomas were fixed in 4% buffered paraformaldehyde and embedded in paraffin. For expression and mutation analysis, pituitary specimens were collected and transferred under aseptic conditions to 2.0 ml cryogenic vials (Corning B.V. Life Sciences, Schiphol-Rijk, The Netherlands), quick-frozen in liquid nitrogen and stored at -70°C until analyzed. As indication for presence of neoplastic tissue, the presence of a POMC-to-GH expression-ratio equal to or higher to that in normal pituitary tissue was used as inclusion criteria for the tissues included in mutation analysis (data not shown).

Immunohistochemistry

Pituitary tissue specimen from one normal canine pituitary gland and 12 pituitary adenomas was immunolabelled and developed as described previously.[9] Antibodies were used as follows: rabbit anti-Tpit 1:25,[9] and mouse anti-POMC 1:500 (Cortex Biochem, San Leandro, USA).[21] Secondary antibodies were used 1:150 (Vector Laboratories, Burlington, Canada).

RNA isolation and cDNA synthesis

Total RNA was extracted from control pituitaries and 14 pituitary adenomas according to the manufacturer's instructions with the RNeasy Mini Kit (Quiagen, Leusden, The Netherlands). An on-column DNase digestion step (Quiagen RNase-free DNase kit) was included during RNA purification. RNA was quantified spectrophotometrically using Nanodrop ND-1000 (Isogen Life Sciences, IJsselstein, The Netherlands) and 1 µg was reversed transcribed in a total volume of 20 µl. For the normal pituitaries and 6 adenomatous specimens, cDNA was synthesized by using oligo-T primers and AMV Reverse Transcriptase (Promega, Leiden, The Netherlands). The RT reaction was performed at 42° for 60 minutes. For 8 adenomatous specimens, cDNA synthesis was performed using oligo (dT) primers combined with random hexamer primers and an MMLV-derived reverse transcriptase according to the manufacturer's instruction (iScript cDNA Synthesis Kit, Bio-Rad, Veenendaal, The Netherlands).

Primer design

Primers for Tpit were designed to a sequence that was derived from the canine Boxer genomic DNA sequence available at National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/genome/seq/BlastGen/BlastGen.cgi?taxid=9615) that showed the highest identity to the human Tpit gene. The exon-intron organization of the canine Tpit gene was deduced by comparison with the human TPIT cDNA (NM005149.1). Three intron-spanning primer-pairs were developed with PrimerSelect software version 5.05 (DNASTAR Inc., Madison, WI), to cover the deduced 8 exons of the gene (Table 1). For sequence analysis of genomic DNA, a forward primer (5'-TGAGCGAGCTGGGCATCC-3') located in exon 1 and a reverse primer (5'-GGGGGCTTCATCCTCTACTTCTC-3') located in intron 1 were designed.

Table 1
Nucleotide sequences of canine Tpit-specific primers for RT-PCR

Primer pair	F/ R	Sequence	Ta (ºC)	Product size (bp)	Exons
1	F	5'-AGCAGGCAAGTGAGAGGAGGAAGG-3'	65	690	5'UTR-4
	R	5'-AGCTGTTATCTCCTCATTCTG-3'			
2	F	5'-AGCCCCCATCTCCTTCA-3'	58	990	2-3'UTR
	R	5'-ATGGGGTTTCTAAATGCTCTAC-3'			
3	F	5'-CACATAGTGCGTGTTGGAGG-3'	65	508	3-7
	R	5'-CAGACAGGATGCTGGTGTGG-3'			

bp: base pairs; F: forward primer; R: reverse primer; Ta: annealing temperature; UTR: untranslated region

RT-PCR

PCR reaction was performed on 1 µl cDNA in a 50-µl volume containing 1 µl cDNA, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of each primer, 2.5 U Recombinant Platinum Taq polymerase (Invitrogen, Breda, The Netherlands) in 1 x PCR Reaction Buffer (Invitrogen, Breda, The Netherlands). The PCR program consisted of an initial activation at 94°C for 4 min followed by 35 cycles each of 1 min at 94°, 1 min at 58-65° (depending on primers), 1 min at 72°C, followed by a final extension at 72° for 10 min. The products were visualized on ethidium-bromide containing 1.5% agarose gels. For sequence analysis of exon 1, the protocol described cDNA was used with cDNA replaced by 5 ng gDNA as template. Annealing was performed at 58°C.

DNA sequence analysis

For the DNA sequence analysis, the PCR products were diluted 1:10 in distilled water and 1-2 µl of the dilution was used in the tercycle reaction using BigDye Terminator Cycle Sequencing Ready mix (Applied Biosystems, Foster City, CA), according to the standard protocol. The tercycle reaction consisted of 25 cycles each of 30 sec at 96°, 15 sec at 50-53° and 2 min at 60°C. The Tercycle product was purified using multiscreen 96-well filtration plate (Millipore, Amsterdam, The Netherlands), and analyzed on an ABI3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

The DNA sequences were aligned with the Seqman software (DNASTAR Inc., Madison, WI). The canine coding sequence for Tpit was compared with that of human (Accession

number, NM 005149) and mouse (Accession number, AF 348321) with MegAlign software (DNASTAR Inc., Madison, WI), using the ClustalW (Slow/Accurate, Gonnet) method.

Results

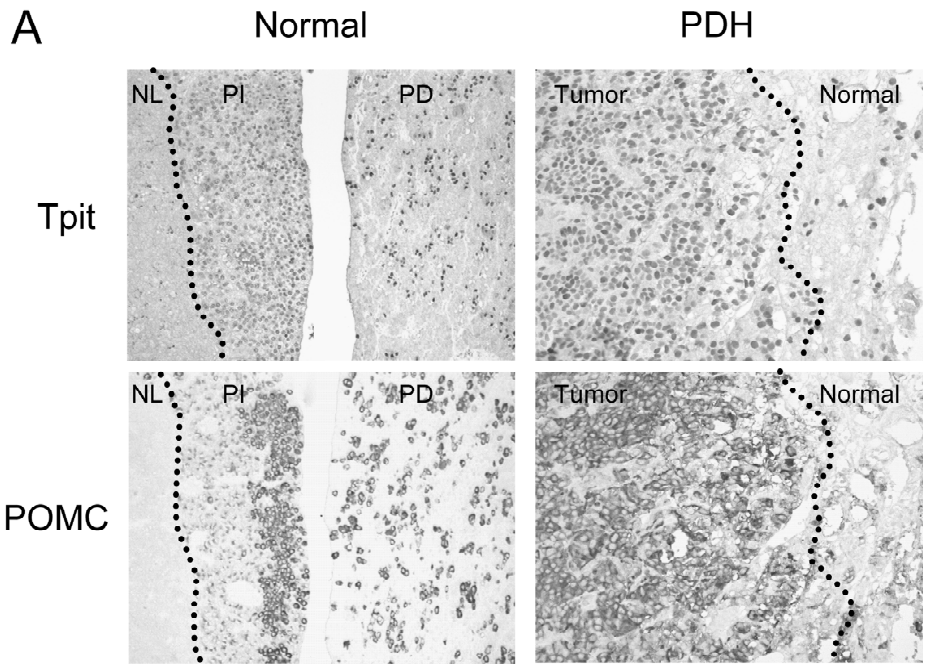
Spatial localization of Tpit protein in the canine pituitary gland

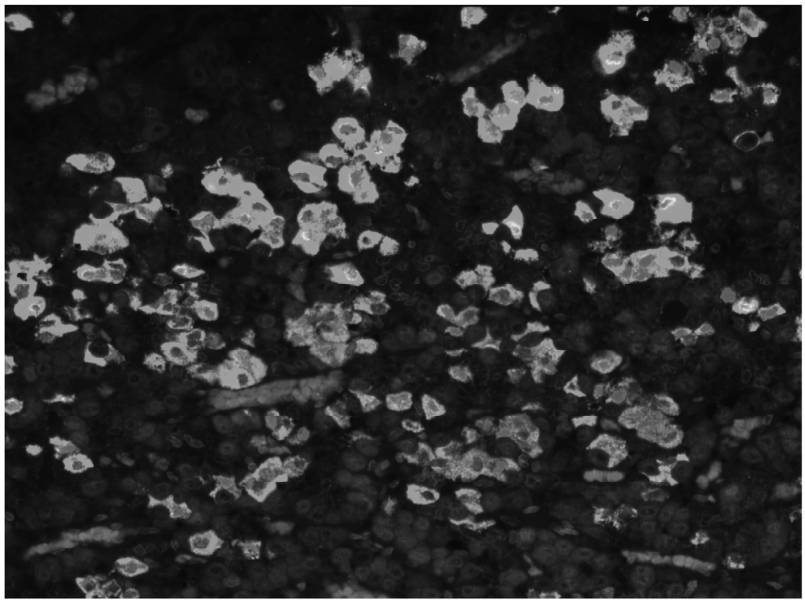
On immunostainings of normal pituitary tissue, Tpit showed nuclear staining which co-localized with POMC in both *pars distalis* and *pars intermedia adenohypophysis* (Figure 1). Thus, Tpit is expressed in canine corticotrophs and melanotrophs. In previous reports, Tpit was shown to be an excellent marker of corticotroph adenomas.[1,21] In the adenomatous dog pituitaries, both neoplastic and normal corticotrophs stained positive for Tpit. Consequently, Tpit expression is a conserved marker for corticotroph cells.

Tpit expression and DNA sequence analysis

Canine Tpit mRNA was expressed in all normal and adenomatous canine pituitary tissues as determined by RT-PCR analysis (Figure 2). The Tpit cDNA sequence was submitted to the GenBank database under accession AY745240. The translated protein was 93.5% identical to human Tpit, 90.8% identitical to murine Tpit (Figure 3), and 57.5% identical to the closely related canine Brachyury (T). No tumor-associated mutation was found by analysis of Tpit cDNA sequences derived from adenomatous tissue. Two silent SNPs in exon 6 were discovered in 2 corticotroph adenomas. In one pituitary tumor a missense SNP (V63M) was found located in the T-box. The pituitary tumor was heterozygous for the SNP and the same heterozygosity was found in the genomic DNA from the same dog. Of 24 healthy dogs of the same breed (Bernese Mountain dogs), 7 dogs were heterozygous and 2 dogs were homozygous for the 63M allele (Figure 4).

Figure 1. (A) Immunohistochemical analysis of pituitary T-box transcription factor Tpit on a normal canine pituitary gland (left) and pituitary specimen from a dog with pituitary-dependent hyperadrenocorticism (PDH) caused by a corticotroph adenoma (right). The nuclear immunoreactivity of Tpit co-localizes with cytoplasmic pro-opiomelanocortin (POMC) in the corticotrophs and melanotrophs of normal *pars distalis* (PD) and *pars intermedia* (PI) *adenohypophysis* and in the pituitary specimen from a dog with PDH. NL indicates the neural lobe. Immunolabelling was performed with ABC-HRP technique, development with DAB (brown), and counterstaining with methyl green. (B) Colocalization immunofluorescent analysis of POMC (green) and Tpit (red) in a representative area of the PD.


A
Normal
PDH
Tpit
POMC
NL
PI
PD
Tumor
Normal
NL
PI
PD
Tumor
Normal


B

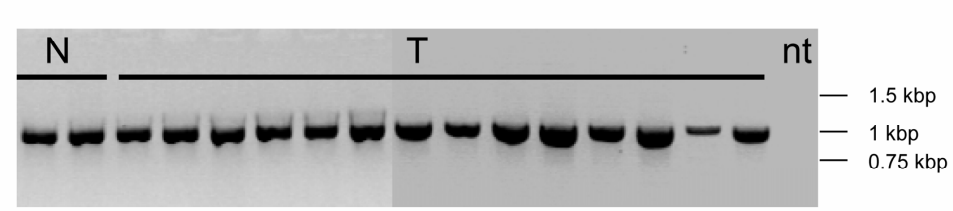


Figure 2. Expression of Tpit in two samples of normal canine *pars distalis adenohypophysis* (N) and pituitary adenoma tissue from dogs with pituitary-dependent hyperadrenocorticism (T), non-template control (nt). The bands represent amplified 990 base pair RT-PCR products of primer pair 2 (Table 2) visualized on a ethidium-bromide containing agarose gel. Size markers are 750, 1000 and 1500 bp. Integrity was confirmed by sequencing.

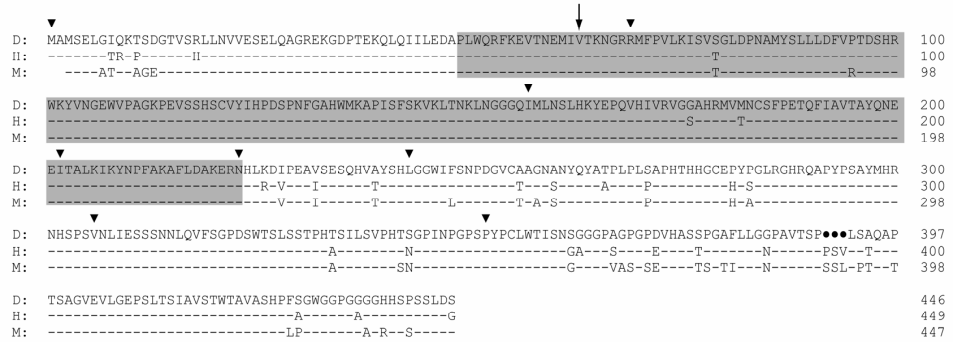


Figure 3. Alignment of canine (D), human (H) and mouse (M) Tpit proteins. Arrowheads indicate the first amino acid in each exon, the filled dots a three-amino-acid gap in the canine Tpit protein, the gray-boxed region corresponds to the T-box, and the arrow indicates the V63 allele (Figure 4).

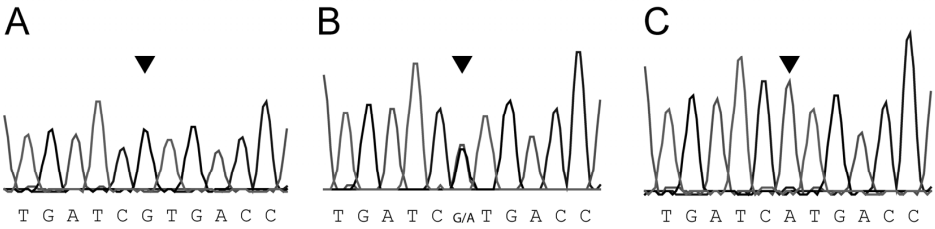


Figure 4. DNA sequences of A) a wild type dog B) a heterozygote dog and C) a dog homozygote for V63M missense single nucleotide polymorphism in the T-box region of the canine Tpit protein.

Discussion

In the present study we report that the POMC-expressing pituitary cell lineages, the corticotrophs and melanotrophs of the canine pituitary gland stained immunopositive for Tpit. Adenomatous POMC-positive corticotrophs and also non-neoplastic corticotroph cells adjacent to a corticotroph adenoma with suppressed immunopositivity for ACTH were immunopositive for Tpit confirming earlier findings in dog and humans.[1,21] Therefore, Tpit is a valuable marker for corticotroph and melanotroph cells in the canine pituitary gland. Tpit is an obligate factor for POMC-transcription[9] and the mutations of Tpit so far published are associated with loss-of-function and early-onset hypoadrenocorticism in humans and mice.[15] Based on the involvement of Tpit in the differentiation of the POMC-producing cell lineage during embryogenesis,[9,16] we hypothesized that a gain-of function mutation in the Tpit gene may take part in the tumorigenesis of canine corticotroph adenomas. However, no tumor-specific mutation in the Tpit gene was found in the analyzed adenomatous tissue specimens. This is in agreement with the findings in a similar study, performed on 8 human corticotroph macroadenomas.[3] Consequently, a gain-of-function mutation of Tpit is less likely to play a major role in the general pathogenesis of canine corticotroph adenomas. Interestingly, we here present a missense mutation in the T-box which is not associated with early onset pituitary ACTH deficiency.

It is concluded that the Tpit protein can be used as a marker for corticotrophs and melanotrophs in both the *pars distalis* and the *pars intermedia adenohypophysis* of the normal and transformed canine pituitary gland. The canine Tpit gene is highly identical to that of other species and mutations in the Tpit gene in canine corticotroph adenomas are uncommon.

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CHAPTER 8

Differential expression of neurogenic differentiation 1 (NeuroD1) in the canine pituitary gland and corticotroph adenomas

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Abstract

Pituitary-dependent hyperadrenocorticism (PDH) in dogs is caused by a pituitary corticotroph adenoma that secretes excess of adrenocorticotrophic hormone (ACTH) and in some cases also α-melanocyte-stimulating hormone (α-MSH). Although PDH is a common disorder in the dog, little is known about the underlying pathogenesis. ACTH and α-MSH are products of posttranslational splicing of the same precursor molecule, pro-opiomelanocortin (POMC). The corticotrophs and melanotrophs have the transcription factor Tbx19 in common, but differ in the presence of the basic helix-loop-helix transcription factor NeuroD1, that is expressed in corticotrophs but not in melanotrophs in mice. The expression of NeuroD1 during pituitary organogenesis promotes corticotroph differentiation and its expression in other epithelial tumors has been associated with more aggressive behaviour. The expression of NeuroD1 has not been studied in the canine pituitary gland. The aim of this study was to quantify the expression of NeuroD1 in the normal canine pituitary gland and in adenomatous pituitary tissue from dogs with PDH. NeuroD1 expression was significantly lower in the neurointermediate lobe than in the *pars distalis adenohypophysis*. NeuroD1 expression varied considerably between the adenomas studied. When normalized by the expression of Tbx19, the expression of NeuroD1 was lower in the adenomas than in the normal *pars distalis adenohypophysis*.

Pituitary-dependent hyperadrenocorticism (PDH), called Cushing's disease in humans, is a common endocrinopathy in the middle-aged or eldery dog. PDH is caused by a pituitary adenoma characterized that secretes excess adrenocorticotrophic hormone (ACTH) and in some cases also α-melanocyte-stimulating hormone (α-MSH). Although PDH is a common disorder in the dog, little is known about the underlying pathogenesis. ACTH and α-MSH are products of posttranslational splicing of a precursor molecule, the pro-opiomelanocortin (POMC). The corticotrophs secrete mainly ACTH, the melanotrophs mainly α-MSH. Spatially, corticotrophs are located in the *pars distalis adenohypophysis* (pars distalis), and most melanotrophs in the *pars intermedia adenohypophysis* (intermediate lobe, pars intermedia). However, there are also corticotrophs in the intermediate lobe and melanotrophs in the pars distalis. The corticotrophs and melanotrophs share a specific transcription factor, the Tbx19, but differ in the presence of the basic helix-loop-helix transcription factor NeuroD1, that is expressed in the corticotrophs but not in the melanotrophs.

Neurogenic differentiation factor D1 (NeuroD1) or Beta2 (β2) is a Class II basic helix-loop-helix (bHLH) factor. The members of the HLH family are important proteins for developmental processes. The Class II HLH transcription factors are characterized by tissue specific expression.[17] NeuroD1 is expressed in progenitor cells and differentiated endocrine cells of the pancreas.[5,6] NeuroD1 is also expressed in the neuroectoderm where it plays an important role in the differentiation of certain structures of the central nervous system.[14,15,19] In the pituitary gland, NeuroD1 it is mainly expressed by the corticotrophs,[24] where it promotes expression of POMC. NeuroD1 forms a heterodimer with a Class I bHLH, also called E proteins (e.g., E47/Pan1). The heterodimer binds to the E box of the POMC promoter and promotes POMC transcription. The bHLH heterodimer acts in synergy with pituitary T-box transcription factor Tbx19 that is dependent on the biocid-related homeodomain protein Ptx1.[13] The Pan1 part of the bHLH heterodimer interacts with the Ptx1[23,24] and Tpit,[13] while NeuroD1 is required for DNA sequence recognition of the E-box.[13]

In addition to POMC-transcription enhancement, NeuroD1 is also involved in the early corticotroph differentiation. Although NeuroD1 is not necessary for cell lineage commitment, NeuroD1-deficient mice show a delayed terminal differentiation of corticotrophs. There seems to be a discrepancy in translation of NeuroD1 between the adult corticotrophs of mice and humans. In mice, the NeuroD1 protein was only detected between embryonic day (e) 12.0 and e15.5 and not in the adult gland, although mRNA is still detectable.[24] In humans, NeuroD1 is detected by immunohistology and *in situ* hybridization in the adult pituitary gland, where the majority of the immunopositive cells are corticotrophs.[21] NeuroD1 may be a marker of poor differentiation and its expression is associated with low grade of differenttiation and aggressive features of human prostate cancers,[7] primitive neuroectodermal tumors[26] and poorly differentiated gastric adenocarcinomas.[8]

In dogs with PDH, there are clinical and morphological differences among the ACTH-secreting adenomas.[10,11] Generally, the adenomas are considered to be benign, slow-growing tumors. However, some adenomas show mass expansion and infiltrative behavior and rapid re-growth of tumor mass after surgical removal. About 10-20% of the tumors express α-MSH to an extent that leads to elevated plasma concentrations of this hormone, and therefore may reflect their origin from the intermediate lobe.[2] These tumors usually have a larger size, and are associated with a higher risk of recurrence.[2,10]

It is hypothesized that NeuroD1 may be a marker for a low degree of differentiation of pituitary corticotrophs and that absence of NeuroD1 expression may indicate for a tumor of

intermediate lobe origin. The aim of the present study was to compare the expression of NeuroD1 in the normal pars distalis and the neurointermediate lobe and to investigate the expression of NeuroD1 in adenomateous tissue of 8 pituitary adenomas from dogs with PDH.

Materials and Methods

Animals

Pituitary specimens from 8 dogs (1 Beagle, 1 Bernese Mountain Dog, 1 English Cocker Spaniel, 1 Golden Retriever, 1 Jack Russel Terrier, 1 Labrador Retriever, 1 Maltese, and 1 Vizsla) with PDH were collected at the time of transsphenoidal hypophysectomy (Meij et al 1997). There were 4 female (2 spayed) and 4 male dogs (2 castrated). The median age was 10 years (range, 6-14), and the median body weight was 22 kg (range, 3.7-34 kg) (Table 1). The diagnosis of pituitary-dependent hyperadrenocorticism was based upon the averaged urinary corticoid-to-creatinine ratio in two consecutive morning urine samples combined with a high-dose dexamethasone test, as described earlier.[25,27] Based on the pituitary height-to-brain area (P/B) ratio (normal sized pituitaries, $< 0.31 \times 10^{-2}$ mm^{-1}),[12] all dogs had enlarged pituitaries (median, 0.82; range, 0.44 to 1.25). All dogs went into initial remission after surgery.

Control pituitary tissue was collected from 12 laboratory dogs. The dogs (6 female dogs, 6 male dogs) were 2 Beagle dogs, 1 Greyhound, 7 Labrador retrievers, and 3 Crossbred dogs. The age ranged from 0.5 to 12.5 years and the body weight ranged from 20 to 25 kg. The dogs were euthanized in other experiments which have been approved by the Ethical Committee, Utrecht University, The Netherlands.

Collection of pituitary specimens

Following extraction of the pituitary gland from the skull in the laboratory dogs, the pars distalis and the neurointermediate lobe were separated. In the dogs with PDH, pituitary specimens were collected during pituitary surgery. Pituitary specimens were transferred under aseptic conditions to 2.0 ml cryogenic vials (Corning B.V. Life Sciences, Schiphol-Rijk, The Netherlands), quick-frozen in liquid nitrogen and stored at –70°C until analyzed. There were tissue specimens from 9 neurointermediate lobes, 12 pars distalis and 8 adenomatous pituitary specimens from dogs with PDH. A representative part of the adenomatous pituitary specimen was processed for histopathology and the diagnosis of corticotroph adenoma was confirmed using immunostaining for ACTH and α-MSH.

RNA isolation and cDNA synthesis

Total RNA was extracted from tissue according to the manufacturer's instructions with the RNeasy Mini Kit (Quiagen, Leusden, The Netherlands). An on-column DNase digestion step (Quiagen RNase-free DNase kit) was included during RNA purification. RNA was quantified spectrophotometrically using Nanodrop ND-1000 (Isogen Life Sciences, Ijsselstein, The Netherlands) and 1 µg was reversed transcribed in a total volume of 20 µl. For the normal pituitaries and 6 adenomatous specimens, complementary DNA (cDNA) was synthesized by using oligo-T primers and AMV Reverse Transcriptase (Promega, Leiden, The Netherlands). The RT reaction was performed at 42° for 60 minutes. For 8 adenomatous specimens, cDNA synthesis was performed using oligo (dT) primers combined with random

hexamer primers and an MMLV-derived reverse transcriptase according to the manufacturer's instructions (iScript cDNA Synthesis Kit, Bio-Rad, Veenendaal, The Netherlands).

Primer design

Primers for NeuroD were based on the canine Boxer nucleotide DNA sequence available at the homepage of National Center for Biotechnology Information homologous to the human NeuroD1 gene. Primers were developed with the PrimerSelect software version 5.05 (DNASTAR Inc., Madison, WI) (Table 1). The POMC and NeuroD1 primers were non-intron spanning. The amplified product was verified by sequencing.

Table 1
Nucleotide sequences of primers used for quantitative PCR analysis

Factor	F/R	Sequence	Ta (ºC)
Tpit	F	5'-CATAGCTGTGACTGCCTATC-3'	59
	R	5'-ACATGCTGGCTCTCAGAGAC-3'	
POMC	F	5'-GCCTGCAAGCCCGACCTCTC -3'	62
	R	5'-CTCCGCCCGCCGCCACCTCTTCTT-3'	
NeuroD1	F	5'-GCTCGTGATGCGAATGGCTC -3'	62
	R	5'-CGCCTTCACCATGCACTACC -3'	

F = forward primer; R = reverse primer; Ta = annealing temperature

Quantitative PCR

Quantitative PCR was performed using a Bio-Rad My-IQ detection system (IQ SYBR green Supermix and My-IQ (Bio-Rad, Veenendaal, The Netherlands) according to the manufacturer's instructions, with a final concentration of the primers of 400 nM each, and 0.5 µl cDNA template per reaction. The ribosomal protein S19 (RPS19) and hypoxanthine phosphoribosyltransferase (HPRT) were used as reference genes.[3] As a control for the purity of neurointermediate lobe and tumor specimens, the expression of growth hormone (GH) mRNA was quantified using GH-specific primers published previously.[1] Primers for amplification of Tpit, POMC and NeuroD1 are presented in Table 1. All reactions were performed in duplo. Tpit and POMC were amplified using a 3-step program and ACTH and NeuroD1were amplified using a 2-step program. Optimal annealing temperature was determined experimentally, and the amplified product was confirmed by sequencing. Efficiency for each reaction was determined by dilution of pooled cDNA samples and tested for each replicate run. A non-template control in duplicate was included on each plate. No efficiency was below 95%. Analysis was performed with My-IQ software (Bio-Rad, Veenendaal, The Netherlands).

Calculations and statistics

The cycle threshold (Ct) values of NeuroD1, POMC and GH were normalized to the Ct values of the reference genes and Tpit using the formula described by Muller and co-workers.[20] The normalized expression data were then analyzed with the non-parametric Mann-Whitney test using SPSS software (SPSS Benelux BV, Gorinchem, the Netherlands). Analysis was also performed using the pair wise fixed reallocation randomization test incorporated in the software programme REST-XL.[22] A P-value <0.05 was considered significant.

Results

Purity of the neurointermediate and adenomatous tissues

Purity of the neurointermediate and adenomatous tissues was investigated by quantification of the expression of GH mRNA. When normalized by reference gene expression, the expression of GH was significantly ($P<0.01$) down-regulated in the neurointermediate lobe by a factor 17.0 compared to GH expression in the pars distalis which indicates a proper separation of the lobes (Figure 1a). When normalized by reference gene expression, the expression of GH was significantly ($P<0.001$) down-regulated in the adenomas by a factor 187 compared to GH expression in the pars distalis indicating purity of the tissue (Figure 1A)

Expression of Tbx19

The expression of the corticotroph and melanotroph marker Tbx19 was significantly higher by a factor 7.8 in the adenomatous tissue compared to Tbx19 expression in the pars distalis ($P < 0.05$) (Figure 1B).

Neurointermediate lobe versus pars distalis adenohypophysis

The expression of NeuroD1 in the neurointermediate lobe was significantly ($P<0.01$) down-regulated by a factor of 5.0 compared with NeuroD1 expression in the pars distalis (Figure 1C). The expression of NeuroD1 in the neurointermediate lobe when normalized by Tbx19, was significantly lower ($P<0.001$) (9.5%) than that in the pars distalis (Figure 1D). The expression of POMC was significantly ($P<0.01$) up-regulated by a factor of 2.9 in the neurointermediate lobe compared with POMC expression in the pars distalis (Figure 1E). normalized by expression of RPS19. When normalized to Tbx19, the expression of POMC in the neurointermediate lobe, was significantly (141.3%) ($P<0.05$) higher than the POMC-expression in the pars distalis (Figure 1F).

Tumor versus pars distalis adenohypophysis

When normalized by Tbx19, the expression of NeuroD1 in the adenomas was significantly lower (21.6%) ($P<0.05$) than the expression of NeuroD1 in the pars distalis (Figure 1D), and the expression of POMC in the adenomas was lower (26.7%) than the POMC expression in the pars distalis (Figure 1F).

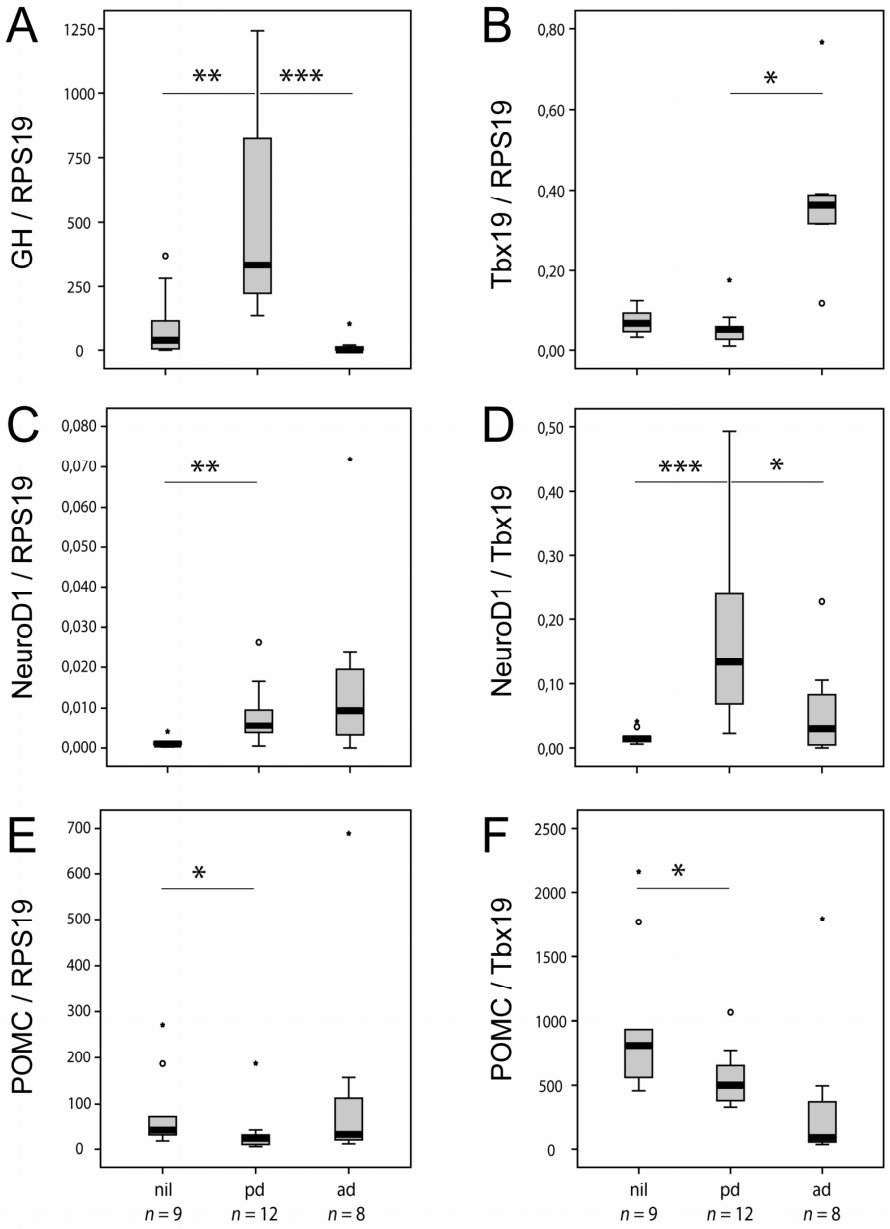

Figure 1. Box-plot graphs representing the expression of growth hormone (GH), T-box transcription factor 19 (Tbx19), Neurogenic differentiation factor 1 (NeuroD1) and pro-opiomelanocortin (POMC) normalized by ribosomal protein S19 (RPS19) and by Tbx19 in the neurointermediate lobe (nil), *pars distalis adenohypophysis* (pd), and corticotroph adenomas (ad). Small circles and stars indicate outliers. *: P<0.05; **: P<0.01; ***: P<0.001.

Expression in the adenomas

The expression of NeuroD1, POMC, and GH in the adenomas is presented in Table 2 which also lists the plasma concentrations of ACTH, cortisol and α-MSH in the individual dogs with PDH. There was a considerable difference in NeuroD1 expression in the adenomas (Figure 2).

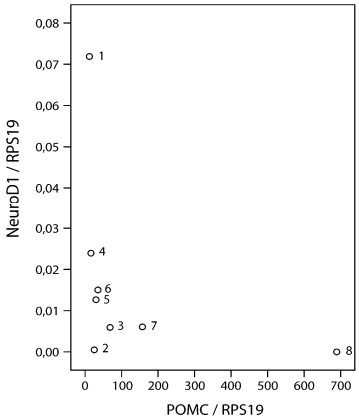

Figure 2. Scatter-plot graph of the expression of NeuroD1 versus that of POMC in 8 canine ACTH-producing macroadenomas.

Discussion

In the present study we show that the NeuroD1 is mainly expressed in the pars distalis of the canine pituitary and that the expression of NeuroD1 in the neurointermediate lobe is low. Our findings are in agreement with those previously reported in mice.[24] However, in the mouse pituitary gland, the NeuroD1 protein is only detected between e12.0 and e15.5 and not in the adult gland, although mRNA is detectable.[24] On the contrary, in human adult pituitary glands, NeuroD1 has been detected both by immunohistology and *in situ* hybridization, with a principal co-localization with the corticotrophs.[21] The difference in protein detection may be ascribed to a species difference in translation activity or to a difference in sensitivity in the methods used in the studies.

The expression of NeuroD1 in adenomatous tissue of dogs with PDH was not different from that in the pars distalis of control dogs, when normalized by the reference genes RPS19 and HPRT. If normalized by Tbx19, the expression of NeuroD1 was lower in the adenomas than in the pars distalis of control dogs indicating that the expression per adenoma cell is lower than that in normal corticotroph. However, there is also a low expression of NeuroD1 in the gonadotrophs of the murine pituitary.[24] Therefore, when normalized by Tbx19, expression of NeuroD1 in the gonadotrophs will be added to that of the corticotrophs, which in turn will be overestimated.[24] This phenomenon may contribute partly to the difference that is seen between the expression of NeuroD1 in the pars distalis and the adenomatous material.

With regard to NeuroD1 expression, the adenomas were a heterogenous group of tumors. A low expression of NeuroD1 and high expression of POMC may indicate an adenoma originating from the pars intermedia as seen in dog 8. It may also be speculated that a low expression of NeuroD1 may indicate a STAT3 activation mediated by cyclin1 repression of

NeuroD1 transcription.[4,16] For example, activation of the leukemia inhibitory factor receptor (LIFR) includes the STAT3 pathway, and we have recently demonstrated that both corticotrophs and melanotrophs of the normal canine pituitary as well as adenomatous tissue of dogs with PDH express LIFR.[9]

Expression of NeuroD1 has been detected in the murine corticotroph tumor cell line (AtT20).[24] In humans, expression of NeuroD1 was confirmed qualitatively in 3 out of 3 human corticotroph adenomas, and immunohistologically in 10 out of 10 ACTH secreting adenomas [21]. In addition, a case report of a human patient with Cushing's disease reported concomitant expression of ACTH and GH in the same cells. Both Pit-1 and NeuroD1 were expressed in many of the adenoma cells.[28] In an RT-PCR analysis of carcinoid tumors associated with ectopic-ACTH syndrome there was a considerable difference in the expression of NeuroD1. About 50% of the tumors were positive for NeuroD1, irrespective if the carcinoids were immunopositive for ACTH or not. It was concluded that NeuroD1 is not a major determinant for ACTH production in these cells, however the expression of NeuroD1 was not related to the clinical outcome.[18]

It is concluded that NeuroD1 in the normal pituitary gland is predominantly expressed in the pars distalis and that the expression in neurointermediate lobe is low. There was a considerable variation in expression of NeuroD1 within the adenoma group. When normalized by the expression of Tbx19, the expression of NeuroD1 was lower in the adenomas than in the normal pars distalis. Additional research including protein analysis is needed to determine whether the expression of NeuroD1 can be related to pituitary cellular origin (corticotrophs or melanotrophs) or clinicopathological characteristics.

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CHAPTER 9

Expression of leukemia inhibitory factor (LIF) and LIF-receptor in the canine pituitary gland and corticotroph adenomas

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Abstract

Leukemia inhibitory factor (LIF) is an important factor for corticotroph cell lineage differentiation and for expression and secretion of adrenocorticotropic hormone (ACTH) in the adult pituitary gland. The aim of this study was to investigate the expression of LIF and LIFRα mRNA and protein in the normal canine pituitary and in corticotroph adenomas, and to perform a mutation analysis of LIFR.

The LIF and LIFR proteins were localized by immunohistochemistry in pituitary glands of control dogs and in adenomatous tissue collected through hypophysectomy in dogs with pituitary-dependent hyperadrenocorticism (Cushing's disease). Quantitative expression analyses were performed for LIF and LIFR, and cDNA from adenomateous tissue from 14 dogs with corticotroph adenomas was screened for mutations.

There was a low magnitude of LIF expression in *pars distalis adenohypophysis* (pars distalis) of control and in tumor tissue. Immunoreactivity in the *pars intermedia adenohypophysis*) (intermediate lobe, pars intermedia) was limited to a few positive cells. In contrast to this, LIFRα was highly expressed in the intermediate lobe. In the pars distalis the LIFR co-localized with ACTH. Immunoreactivity of LIFRα was preserved in corticotroph adenomas and adjacent non-tumorous cells of pars intermedia. Surprisingly, a nuclear/perinuclear immunoreactivity to LIFR was seen in non-neoplastic cells of pars distalis in the presence of a corticotroph adenoma. No mutation was found on mutation analysis of the complete LIFRα cDNA.

These data show that LIFRα is highly co-expressed with ACTH and α-MSH in canine control pituitaries and corticotroph adenomas and that nuclear immunoreactivity for LIFRα in non-tumorous cells of pars distalis may indicate presence of a corticotroph adenoma.

In the dog, pituitary-dependent hyperadrenocorticism (PDH), in humans called Cushing's disease, is a commonly encountered endocrinopathy, caused by a corticotroph adenoma.[44] These adenomas are generally characterized as benign, slow growing pituitary tumors with increased ACTH secretion and reduced sensitivity to glucocorticoid feed-back.[7,25,44] The underlying pathogenesis is still not known. In the present study the candidate genes leukemia inhibitory factor (LIF) and its receptor are being studied. As its name indicates, LIF inhibits the proliferation of leukemias, but acts as a growth factor for several neoplasms.[6,35] For example, LIF stimulates breast cancer proliferation [21] and the proliferation of multiple myelomas.[58]

Leukemia inhibitory factor (LIF) is multifunctional glycoprotein cytokine belonging to the interleukin- 6 (IL-6) family of hemato- and neuropoeietic cytokines.[6,18,22,37,41] In human pituitaries, LIF is expressed in the normal adult, fetal as well as in tumorous tissues [5] where it has important neuroimmune mediating effects on the hypothalamic-pituitary-adrenal axis. The leukemia inhibitory factor induces POMC transcription and ACTH secretion in the corticotroph cells where LIF also acts synergistically with CRH.[2,3,5,10,42,43,46,48] LIF promotes corticotroph cell differentiation and, in this case, counteracts CRH's mitogenic effects.[48] Also, during embryogenesis, LIF has a stimulatory effect on corticotroph cell differentiation,[4,56] and early pituitary directed transgenic overexpression of LIF results in corticotroph hyperplasia and Cushingoid symtoms.[56]

The LIF receptor (LIFR) belongs to the hematopoietic cytokine receptor family, which is a member of the immunoglobulin superfamily.[22,56] Among the hematopoeitins, LIF belongs to the interleukin-6 (IL-6) family, of which all family members shares a signal transducing protein in common, the gp130.[1,12,22,50,56] There are two forms of LIFR, the membrane-bound form LIFRα and a soluble form sLIFR, which has antagonistic effects.[31,57] When the LIFRα binds to its ligand, the LIF-LIFRα complex heterodimerizes with gp130 which is a signalling transducing protein shared by all members of the IL-6 family.[1,12,22,50,56] The LIFR activates several signalling pathways in different cell types, including the Janus protein tyrosine kinase (Jak)- signal transducer and activator of transcription (STAT3), the mitogen-activated protein kinase pathway, and the phosphoinositol 3-kinase pathway.[6,16] In the pituitary, the LIFR-gp130 heterodimer activates POMC gene transcription through the Jak-STAT signal pathways requiring STAT1 and STAT3.[9-11,42] This activation appears to involve both direct binding of the POMC promoter by STAT proteins and a DNA-binding independent mechanism.[10,38] Recently, it was shown that STAT reverses glucocorticoid-dependent POMC gene inhibition.[30]

LIF has been detected in both human and murine pituitaries, and LIFR transcripts has been detected in human pituitary adenomatous tissue.[26,46] However, presence of the protein has only indirectly been demonstrated through demonstration of LIF-binding sites on the surface of cultured human pituitary cells[5]. The expression of LIF in the canine pituitary has so far not been studied in the dog. With the hypothesis that LIF and its receptor may play a role in the pathogenesis of corticotroph adenomas, we present here a study on LIF and LIFR in canine pituitaries and corticotroph adenomas including immunoreactivity for LIF and LIFR, qPCR expression profiles and a mutation analysis for LIFR in canine corticotroph adenomas.

Materials and Methods

Tissue

Pituitary specimens from 24 dogs with PDH were collected at the time of transsphenoidal hypophysectomy[33,34] for the treatment of PDH. There was 1 Beagle, 1 Bearded Collie, 1 Bernese Mountain Dog, 1 Boxer, 2 Dachshunds, 1 Dutch Shepherd, 3 English Cocker Spaniels, 1 Golden Retriever, 1 Havanese, 1 Irish setter, 1 Jack Russel Terrier, 1 Labrador Retriever, 1 Maltese, 1 Minature Poodle, 1 Shih Tzu, 1 Siberian Husky, 1 Soft Coated Retriever, 1 Vizsla, 1 Welsh Springer Spaniel, and 2 Crossbred dogs. There were 14 female dogs (9 spayed), 10 males (3 castrated), median age was 9 years (range, 3-12), and median body weight was 17 kg (range, 3.7-48 kg). The diagnosis of pituitary-dependent hyperadrenocorticism was based upon the averaged urinary corticoid-to-creatinine ratio in two consecutive morning urine samples combined with a high-dose dexamethasone test, as described earlier.[45,49] Based on the pituitary height-to-brain area ratio (P/B) (nonenlarged pituitaries, $< 0.31 \times 10^{-2}$ mm^{-1})[29], 21 dogs had enlarged pituitaries. All dogs went into initial remission after surgery.

For immunoreactive studies control pituitary tissue was collected from 2 female Greyhounds (11 and 12.5 years old) and one female and one male Labrador retriever (3.5 and 3 years old). The pituitary from one of the Greyhounds was harboring a corticotroph hyperplasia. For protein-blot analysis control pituitary tissue was used from one 12-year-old, male Greyhound and one 2-year-old Labrador Retriever. For PCR experiments, control pituitary tissue was collected from 13 laboratory dogs. The dogs (6 female dogs, 6 male dogs were 2 Beagle dogs, 1 Greyhound, 7 Labrador retrievers, and 3 Crossbred dogs. The age ranged from 0.5 to 12.5 years and the body weight ranged from approximately 20 to 25 kg. The dogs were euthanized in other experiments which have been approved by the Ethical Committee of the Faculty of Veterinary Medicine, Utrecht University, The Netherlands.

Immunohistochemistry analysis

Pituitary tissue specimen from control pituitaries and pituitary adenomas were fixed in 4% buffered paraformaldehyde and embedded in paraffin. Serial 3 µm-thick sections were mounted on silane-coated slides, deparaffinized in xylene and rehydrated in graded alcohol to PBS (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.4). Slides were blocked for 60 min using 10% normal goat serum (DAKO, Glostrup, Denmark) (LIFR) or 10% normal horse serum (Santa Cruz, Tebu-bio, Heerhugowaard, The Netherlands) (LIF and ACTH) followed by incubation with avidin/biotin blocking kit (Vector Laboratories, Peterborough, UK). The slides were incubated over-night with primary antibodies; anti-human-LIFR rabbit polyclonal antibody (C19, Santa Cruz) (diluted 1:100) which is raised against a peptide mapping at the C-terminus of the receptor that is shown to be highly homologous with the canine LIFR sequence[24]. The anti-human-LIF goat polyclonal antibody (N18, Santa Cruz) (diluted 1:50) was raised against an epitope mapping at the N-terminus of the cytokine. The anti-$ACTH_{1-24}$ was a monoclonal antibody (diluted 1:100) (Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands). Following incubation with primary antibody, slides were reacted with biotin-labeled anti-rabbit, anti-goat and anti-mouse IgG, respectively, incubated with ready-to-use streptavidin-horseradish peroxidase (HRP) (Vector Laboratories) and detected with 3, 3'-diaminobenzidine (DAB) (Vector Laboratories) which was followed by

enhancement with DAB enhancing solution (Vector Laboratories). The slides were counterstained with methyl green (Vector Laboratories), dehydrated, mounted with Vecta-Mount mounting medium (Vector Laboratories) and photographed. The LIF and LIFR antibodies were tested for specificity with the use of a specific blocking peptide (LIFR) and protein-blot analysis. Negative controls were performed by omitting the primary antibody. Immunoscoring was performed by multiplying the score of the stained cells (1-4), staining intensity (1-4) and staining homogeneity (1-4) according to the protocol published by Kontogeorgos and co-workers 2000[24].

Immunofluorescence analysis

Co-localization studies were performed for LIF and LIFR with ACTH. Serial 3 µm-thick sections were mounted on silane-coated slides, deparaffinized in xylene and rehydrated in graded alcohol to PBS (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.4). The slides were transferred to an Antigen Unmasking Solution (Vector Laboratories) and heated in a microwave oven (850W) for 5 min followed by 20 min of cooling down. Blocking was performed during 60 min with 10% normal chicken serum (Santa Cruz) (LIF), 10% normal goat serum (DAKO, Glostrup, Denmark) (LIFR/ACTH). The slides were incubated over-night with anti-LIF (1:50) or anti-LIFR (1:20) antibodies as described above, then washed twice in PBS. For LIF detection, the slides were incubated with the secondary antibody Alexa Fluor 488 chicken-anti-goat IgG (diluted 1:100) (Invitrogen, Breda, The Netherlands), followed by a washing step, blocking with 10% normal goat serum and incubation with anti-ACTH antibodies for 60 min., the slides were then incubated with TOPRO-3 (diluted 1:1000) (Invitrogen) for 30 min For LIFR detection, the slides were incubated with anti-ACTH antibodies for 60 min, and after washing, incubated for 60 min with secondary antibodies, Alexa Fluor 488 goat-anti-rabbit IgG (diluted 1:125) (Invitrogen) and Alexa Fluor 568 conjugated goat-anti-mouse IgG (diluted 1:150) (Invitrogen). After a washing step nuclear staining was performed with TOPRO-3 (diluted 1:1000) (Invitrogen) for 30 min, followed by washing and mounting (Fluorsave, Calbiochem, San Diego, USA). The stained slides were stored in the dark at 4°C and photographed with a Leica TCS SP Confocal Laser Scanning Microscope (Leica Microsystems B.V., Rijswijk, The Netherlands). Single stainings with primary antibody with LIF, LIFR and ACTH, respectively, were performed as controls. Negative controls were performed by omitting the primary antibody.

Protein blot analysis

Pituitary tissue was snap-frozen in liquid nitrogen and stored at –70°C. The pituitary tissue was homogenized in RIPA buffer containing 1% Igepal, 0.6 mM phenylmethylsulfonyl-fluoride, 15 µg/ml aprotinine, and 1 mM sodium-orthovanadate (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands). Protein concentrations were determined using a Lowry-based assay with DC Protein Assay Reagents (Bio-Rad, Veenendaal, The Netherlands). Approximately 20 µg of protein was loaded per well. The supernatant was denatured for 3 min at 95°C and electrophoresed on 10-12% Tris-HCL polyacrylamide gels (Bio-Rad, Veenendaal, The Netherlands). A standard of recombinant proteins were used, Precision Plus Protein Standards (Bio-Rad). The proteins were transferred onto Hybond-C Extra Nitrocellulose membranes (Amersham Biosciences, Roosendaal, The Netherlands) using a

Mini trans-Blot Cell blot-apparatus (Bio-Rad). Immunodetection was based on an ECL Western blot analysis system, performed according to the manufacturer's instructions (Amersham Biosciences). The membranes were incubated with 4% ECL blocking solution in tris buffer saline (TBS) (0.01 mM Tris-HCl, 150 mM NaCl, pH 8.0) supplemented with 0.1% Tween-20 (TBST) (Boom BV, Meppel, The Netherlands) for 1 hour under gentle shaking. The incubation of the primary antibody was performed at 4°C over-night for both antibodies in TBST with 4% BSA (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands). After washing, the membranes were incubated at room temperature for 1 h with their respective HRP-conjungated secondary antibody (1:20 000) (anti-rabbit-HRP for LIFR (R&D systems, Abingdon, UK) and chicken anti-goat-HRP for LIF (Santa Cruz)). The membranes were developed in ECL Advance solution (Amersham Biosciences) and exposed to Kodak BioMax Light-1 films (Sigma-Aldrich Chemie BV). The blots were digitalized using a Canonscan D660U scanner (Canon, Amsterdam, The Netherlands).

RNA isolation and cDNA synthesis

After removal, pituitary tissue was quick-frozen in liquid nitrogen and stored at –70°C. Total RNA was extracted from frozen pituitary adenomatous tissue from 14 dogs with PDH using the RNeasy Mini Kit according to the manufacturer's instructions including the optional on-column DNase digestion (Quiagen, Leusden, The Netherlands). RNA was quantified spectrophotometrically using Nanodrop ND-1000 (Isogen Life Sciences, IJsselstein, The Netherlands) and 1 µg was reverse transcribed in a total volume of 20 µl. For mutation analysis of 6 adenomatous specimens oligo-T primers and AMV Reverse Transcriptase were used according to the manufacturer's instructions (Promega, Leiden, The Netherlands). The RT reaction was performed at 42° for 60 minutes. For mutation analysis of 8 adenomatous specimens, normal tissue, and for all tissue included in the qPCR analysis, cDNA synthesis was performed using oligo (dT) primers combined with random hexamer primers and an M-MLV-derived reverse transcriptase according to the manufacturer's instruction (iScript cDNA Synthesis Kit, Bio-Rad, Veenendaal, The Netherlands). An additional DNase step with TURBO DNA-free (Ambion , Applied Biosystems, Nieuwerkerk, The Netherlands) according to manufacturer's instructions was performed on each sample included in the qPCR analysis.

Mutation analysis

PCR was performed on 1 µl cDNA in a reaction of 1 x supplied reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of each primer, and 2.5 U Recombinant Platinum Taq polymerase (Invitrogen, Breda, The Netherlands), at an experimentally determined optimal annealing temperature for each primer pair, using MiniCycler units (MJ Research Inc, Bio-Rad, Veenendaal, The Netherlands). The products were visualized on ethidium-bromide containing 1.5% agarose gels. Previously published primers covering the whole coding sequence for canine LIFR were used for sequencing analysis[24]. Additionally, two primer pairs were developed using PrimerSelect software version 5.05 (DNASTAR Inc., Madison, WI); 5'-ACTGACTACTTTTGCACGGATGAT-3' and 5'-AACCCCTGTCATTCCA-CTTT-3' were used for additional amplification of the 5'end of LIFR cDNA. The non-intron-spanning primers 5'-TCTGATGCGGAAGCTGAGAA-3' and 5'-GAGCTCACTG-

AGATGGCAGA-3' were used for complementary amplification of the 3'end of LIFR. The amplified products were verified by sequencing.

The PCR products were diluted 1:10 in distilled water and 1-2 µl of the dilution was used in the sequence reaction using BigDye Terminator Cycle Sequencing Ready reaction (Applied Biosystems, Foster City, CA), according to the standard protocol. The tercycle reaction consisted of 25 cycles each of 30 sec at 96° C, of 15 sec at 50-53° C and of 2 min at 60°C. The Tercycle product was purified using multiscreen 96-well filtration plate (Millipore, Amsterdam, The Netherlands), and analyzed on an ABI3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Mutation analysis was performed with the Seqman software (DNASTAR Inc., Madison, WI).

Quantitative PCR

Quantitative PCR was performed using a Bio-Rad My-IQ detection system (IQ SYBR green Supermix and My-IQ (Bio-Rad, Veenendaal, The Netherlands) according to manufacturer's instructions, with a final primers-concentration of 400 nM each, and 0.5 µl cDNA template per reaction. LIF was amplified using a 3-step program and LIFR was amplified using a 2-step program. Optimal annealing temperature was determined experimentally, and the amplified products were confirmed by sequencing as described above. Efficiency for each reaction was determined by dilution of pooled cDNA samples and tested for each replicate run. No efficiency was below 90%. Analysis was performed with My-IQ software (Bio-Rad, Veenendaal, The Netherlands). The LIF primers used were non-intron-spanning 5'-GAGCCCCCTTCCTATCAC and 5'- CCAGCCGGGTCTTCTCC-3' located in exon 3. For LIFR an intron-spanning primer-pair, 5'-ACTGGAGTTGGACCTCAGAC-3' located in exon 6 and 5'-CTGAGAATCAGGTGACCAAG-3'located in exon 6 and 7.[24] The ribosomal protein S19 and hypoxanthine phosphhoribosyltransferase (HPRT) were used as reference genes.[13] Additionally, the pituitary-specific T-box Tbx19 (Tpit) was used as an marker for pro-opiomelanocortin (POMC) expressing cells (corticotrophs and melanotrophs.[23,53] Presence of high POMC expression in relation to GH expression in the adenomatous material was confirmed with quantitative PCR (data not shown).

The cycle threshold (Ct) values of LIF and LIFR were normalized to the Ct values of the reference genes, using the formula described by Muller and co-workers[36]. The expression of LIF was normalized to RPS19 and HPRT and the expression of LIFR was normalized to RPS19, HPRT and Tpit was used as reference genes. The normalized expression data were then analyzed using SPSS software (SPSS Benelux BV, Gorinchem, the Netherlands). For data that were not normally distributed, the non-parametric Mann-Whitney test was used.

Results

Spatial localization of the LIFR and LIF proteins

The immunoreactivity for LIF was diffusely cytoplasmatic. In control pituitary tissues, most cells of the pars distalis were immunopostitive for LIF protein to a varying but mainly low degree of intensity, and only partially co-localized with ACTH. (Figure 1A, D). In the pars tuberalis a perinuclear staining pattern was seen. The pars intermedia was almost completely immunonegative for LIF except for a few positive cells (Figure 1B). The distribution and degree of staining intensity varied greatly in corticotroph adenomas (score 1-32), in 4/13

adenomas LIF, was almost undetectable. Most tumors (9/13) showed heterogenous distribution of LIF with variable staining intensity and clusters of adenoma cells with stronger LIF immunoreactivity. In one tumor, LIF showed an intranuclear staining pattern. The immunoreactivity of the non-tumorous tissue, if available, showed an equal or higher staining intensity than the tumorous tissue. Thus, only a subpopulation of the ACTH positive tumor cells stained positive for LIF (Figure 1E, F).

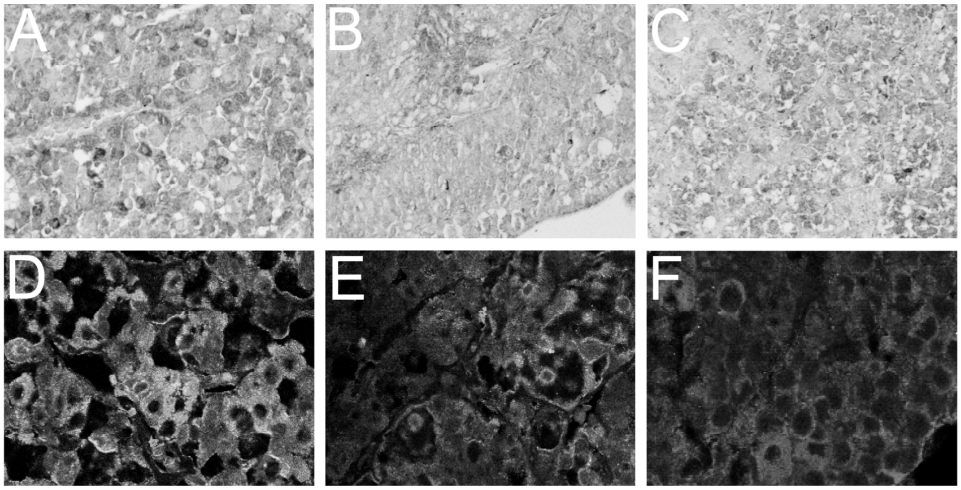

Figure 1. Immunoreactivity to leukaemia inhibitory factor (LIF) detected with DAB (brown) in the *pars distalis adenohypophysis* (A), and *pars intermedia adenohypophysis* (B) of a control dog, and a pituitary adenoma (C). Counterstaining was performed with methyl green. Immunofluorescence reaction for LIF (green) and ACTH (red) in the *pars distalis adenohypophysis* of a control dog (D), and a corticotroph adenoma infiltrating normal tissue (E) and tumerous tissue from a second corticotroph adenoma (F) (Magnification 400 x).*LIFR*

The LIFR was strongly co-expressed with the POMC-expressing cells in both the pars distalis and the pars intermedia (Figure 2, 3). In pituitaries harboring an adenoma, the expression of LIFR followed that of POMC (Figure 4). Non-tumorous cells of the pars distalis were immunonegative, but parts of the pars intermedia were still positive, similar to what was seen for POMC. Additionally, an intranuclear to perinuclear immunoreactivity for LIFR was present in non-tumorous pituitary cells of the pars distalis in all (10/10) available tissue specimes from PDH dogs (Figure 4). This was not observed in the normal control tissues or in the pituitary adenomas with corticotropic hyperplasia. One dog showed both low LIF and LIFR.

Protein blot analysis

The calculated molecular weight of the LIFR protein is 124 kDa. The mature forms of both mouse and human LIF are highly glycosylated molecules with the glycosylation moyeity varying in molecular weight from 38-67 kDa.[6] The expected size of LIFR on protein blot was

about 190 kDa.[1] Additional specific immunoreactive bands were seen at the size of 80 and 90 kDa (Figure 5).

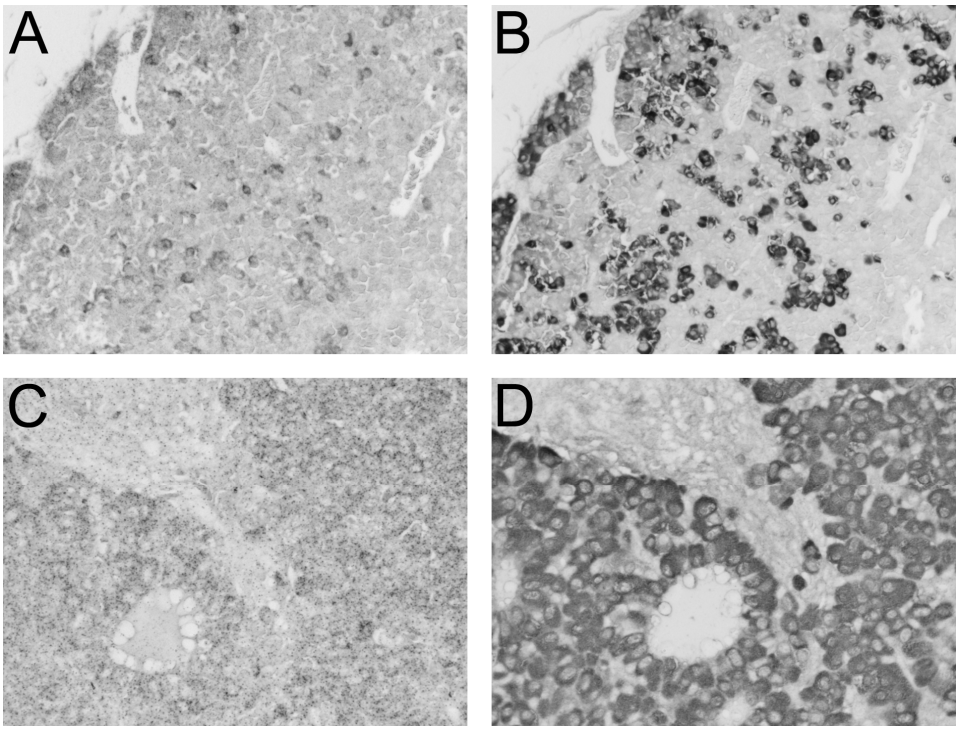

Figure 2. Immunoreactivity detected with DAB (brown) for leukemia inhibitory factor (LIFRα) (A and C) and adrenocorticotropic hormone (ACTH) (B and D) in the *pars distalis adenohypophysis* (A and B) and the *pars intermedia adenohypophysis* (B and D) of a control dog (Magnification 400 x).

RT-PCR reaction and mutation analysis

LIFR was expressed in all 14 adenomatous tissues investigated. No mutations were found on full-length cDNA mutation analysis of 14 corticotroph adenomas. However, a few heterozygote SNP's were found that did not result in different amino acids. When BLASTed to the canine genome available at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST), the sequence of the canine LIF gene is located at chromosome 26 in close vicinity to the oncostatin M (OSM) gene which was identified by entering the human OSM mRNA sequence into the Basic Local Alignment Search Tool (BLAST) available for the dog-genome at the NCBI homepage.

Quantitative PCR

The HPRT and RPS19 were first tested for stability of expression in the pituitary gland, that earlier has been shown for other canine tissues.[13] The presence of tumorous tissue in the pituitary sample was analyzed with qPCR to POMC and GH expression. Only tissue samples with a high POMC to GH relation were included in the qPCR study (data not shown).

The expression of LIF in the pituitary was in general low. There was no detectable LIF in 4 out of 14 adenomas, 4 out of 12 normal pars distalis and in 1 out of 9 neurointermediate lobes. The highest values of normalized expression of LIF were measured in pituitary adenomas. However, the expression of LIF was not significantly different between the groups (Figure 7A).

The expression of LIFR normalized to Tbx19 expression was significantly higher in the normal pars distalis than that in the neurointermediate lobe and in the corticotroph adenomas (Figure 7B).

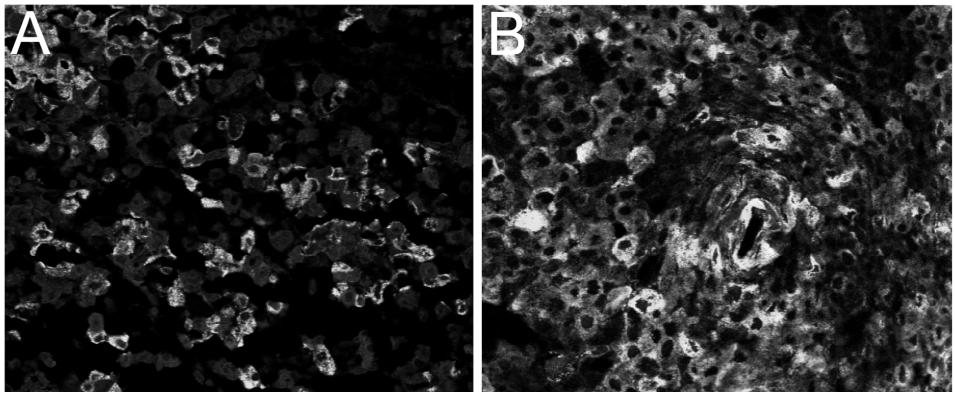

Figure 3. Immunofluorescence double-staining with leukemia inhibitory factor receptor α (LIFRα) (green) and adrenocorticotropic hormone (ACTH) (red) of the *pars distalis adenohypophysis* of a control dog (A) and a corticotroph adenoma (B) from a dog with pituitary-dependent hyperadrenocorticism (Cushing's disease). Co-localization gives a yellow staining (Magnification 800 x).

Discussion

This report shows that the expression of LIFRα is highly specific for POMC-expressing cells of the canine pituitary gland and corticotroph adenomas, and that a presence of a corticotroph adenoma is indicated by a nuclear immunoreactivity to LIFRα in non-tumorous cells of the pars distalis.

LIF

The expression of LIF was dispersed evenly throughout the pars distalis, and was only partly co-localized with ACTH, which is in agreement with previous findings on fetal pituitary glands (20 wk gestation) where LIF co-localizes with 30% of ACTH expressing cells, about 20% of GH expressing cells (somatotrophs) and about 15% of non-hormone expressing cells.[5] The expression of LIF was generally low in the pars distalis and surprisingly low, almost completely absent in the pars intermedia. The intensity of LIF staining varied considerably among the adenomas, of which a few showed a high expression

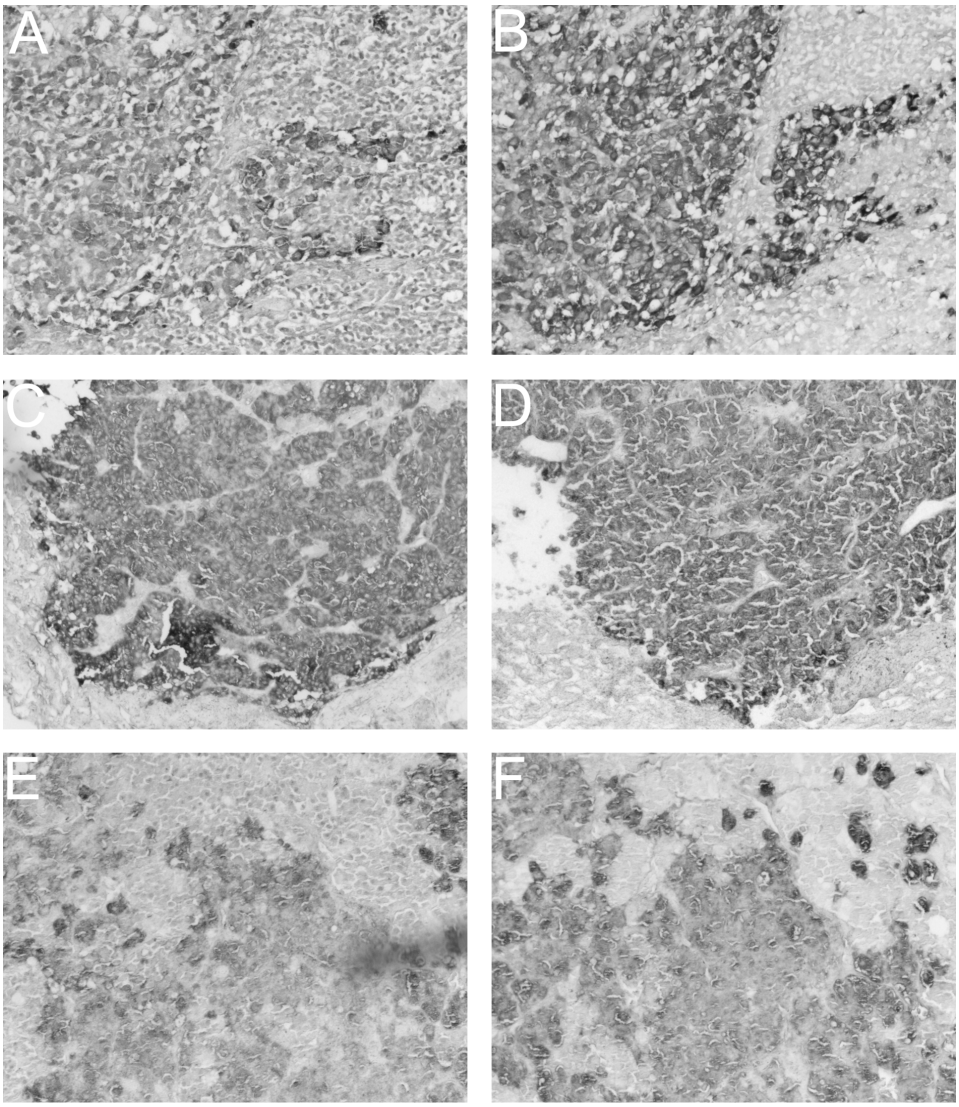

Figure 4. Immunoreactivity detected with DAB to leukemia inhibitory factor receptor (LIFRα) (left) and adrenocorticotropic hormone (ACTH) (right) in corticotroph adenomas from dogs with pituitary-dependent hyperadrenocorticism (Cushing's disease). The LIFRα co-localises with ACTH. In two specimens (A and E) an intranuclear immunoreactivity can be seen in the adjacent non-tumorous tissue (Magnification 400 x).

in comparison to normal tissue. The heterogenous immunoreactivity for LIF in the corticotroph adenomas is in agreement with previous findings on human corticotroph adenomas by Kontogeorgos and co-workers[28] In that series, 98 pituitary adenomas were investigated including 31 corticotroph adenomas. Of these, only 24 (77.5%) were

immunopositive for LIF protein. However, the staining grade of the corticotroph adenomas was the lowest among the different types of pituitary adenomas.

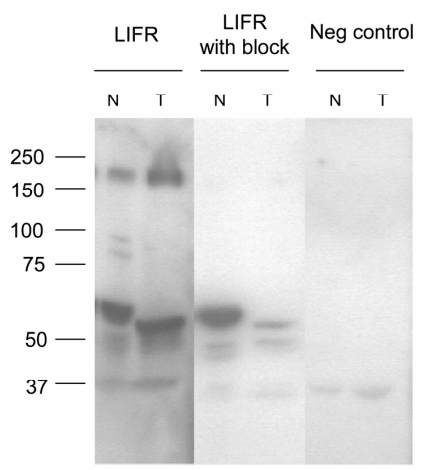

Figure 5. Protein-blot analysis using antibodies directed to leukemia inhibitory factor receptor (LIFRα) (left). N = *pars distalis adenohypophysis* from a control dog; T=corticotroph adenoma (T). The antibody reactivity was blocked by preincubation with a specific blocking peptide (LIFR with block), and the LIFR antibody was omitted as a negative control (Neg control). The expected size of LIFRα was 190 kDa. Additional two smaller proteins, 80 and 90 kDa, can be seen in the control sample. A smaller, partially blocked, protein is seen at about 50 kDa in the adenoma tissue.

LIFR

The co-expression of LIFR and ACTH seen in the present study is in agreement with the indirect findings made by Akita and co-workers 1995, who sorted pituitary cells by the use of an anti-LIF antibody. These sorted cells consisted almost exclusively of ACTH-secreting cells.[5] However, to the author's knowledge, this is the first time that a direct immunostaining for LIFR has been performed on pituitary tissue in any species.

An explanation for the strong immunoreactivity of LIFR in the intermediate lobe despite an almost complete absence of LIF-expression may be that the cells secrete another cytokine of the IL-6 family e.g., oncostatin M, that make use of the LIFR for signalling transmission. However, the predominant cell type in the intermediate lobe is the melanotroph. The melanotrophs secrete α-MSH,[15] which is a strong anti-inflammatory agent and regulator of the early-phase hypothalamic response to cytokine stimulation.[17,47] Regarding the rich arterial blood supply to the neurohypophysis and the longitudinal vessels running in close apposition to the intermediate lobe, it may be hypothesized that the expression of LIFR on the melanotrophs enables these cells to react on circulating cytokines and thereby take part in a fast-reacting anti-inflammatory response system.[17]

It is well-known that the LIF-LIFR signalling cascade, intracellularly mediated by the Jak-STAT pathways, promotes ACTH expression.[2,3,5,10,42,43,46,48] The distal promoter region of ACTH contains a STAT-binding-site in close vicinity to the Nur-responsive element[30] Down-regulation of the LIFR in normal corticotrophs from pituitaries harbouring a corticotroph adenoma, may therefore contribute to the reduced ACTH expression in these cells. Interestingly, there was a nuclear/perinuclear LIFR-immunoreactivity in non-neoplastic cells of the pars distalis in pituitaries harbouring a corticotroph adenoma. This reactivity disappeared with the use of a specific blocking peptide. This may indicate the presence of a tumor-dependent transformation of the non-tumorous cells of the pars distalis. Also, nuclear localization has been documented for another receptor of the cytokine family, the prolactin

receptor. In this case, the wild-type hPRLr is translocated to the nucleus where it potentiated STAT5a transactivation.[20]

The LIFR is a complex molecule with an intricate regulation of gene transcription. There are two forms of LIFR recognized in human and mouse; a membrane-bound form (LIFRα), and a soluble form (sLIFR). The soluble form lacks the transmembrane and cytoplasmic part of the receptor and is secreted into the circulation where it binds LIF and thereby acts as an antagonist.[31,32,39,52,57] In addition at least two promoter regions have been identified in human cells.[6,54] Our data from the protein-blot assay showed LIFR-immunoreactive proteins of different lengths. A short form of LIFR in the region 50-60 kDa was detected in a pituitary gland tumor. Short forms of LIFR-immunoreactive proteins have previously been reported in

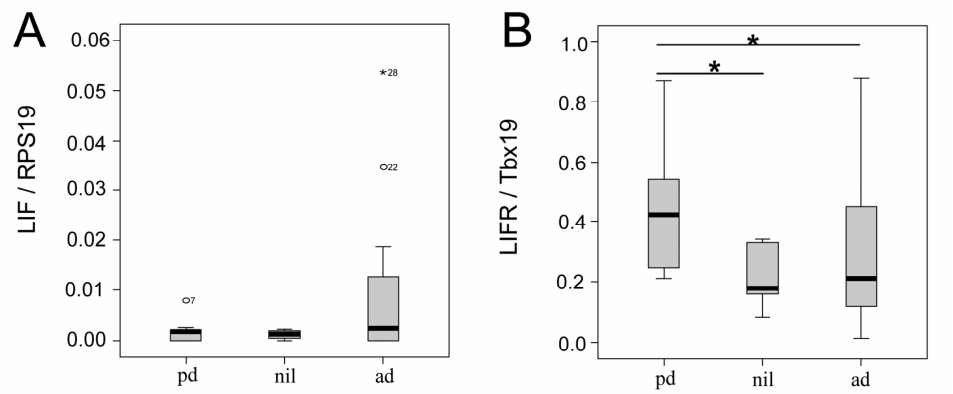

Figure 6. Box-plot graphs representing the expression of leukaemia inhibitory factor (LIF) normalized by the reference gene ribosomal protein S19 (RPS19) (A) and the expression of the LIF receptor normalized by the corticotroph and melanotroph-specific T-box transcription factor Tbx19 (B). The boxes represents expression in the *pars distalis adenohypophysis* (pd), neurointermediate lobe (nil), and canine corticotroph adenomas (ad).

studies of hepatic cells and testicular tissue [8,19]. The interpretations were different. In the hepatic cells, short proteins immunoreactive to LIFRα were interpreted as LIFR degradation products, which increased with cytokine stimulation concurrently with a decrease in the presence of full-length LIFR.[8] If this is the case, presence of a short form of LIFR in corticotroph adenomas indicate the presence of cytokine stimulation. However, no reduction in full-length LIFR was seen. Taken together these findings may suggest that there is a loss of function in the inhibiting mechanism of the expression of LIFRα. Another possibility, in agreement with the interpretation of the findings in the testis,[19] is that there may be a specific expression of a truncated form of LIFRα in parallel to what has been found for the human prolactin receptor.[20]

The expression of LIFR was generally high in both normal and adenomatous tissue, which is in agreement with previous findings.[55] However, when normalized to the expression the corticotroph-specific Tbx19, the expression of LIFR was significantly higher in the pars distalis than in the pars intermedia or adenomas. The mutation-analysis of pituitary tissue from 14 pituitary adenomas revealed no mutations which is in agreement with what has been reported for human pituitary adenomas.[26]

To the author's knowledge, the present report is the first of its kind to present immunoreactivity data for pituitary tissue using antibodies directed to LIFR. There was a striking co-localization between LIFR and the POMC. It has been recognized that STAT-signalling pathways are important for tumor biology.[27] Retinoic acid inhibits LIF-signalling pathways in mouse embryonic stem cells by down-regulation of LIFR[51] and by interaction with the nuclear receptors Nur77 and Nurr1.[40] Recently, it has been presented that retinoic acid reduces plasma concentrations of ACTH in dogs with PDH [14]. Therefore, taken together, our findings and those of previous studies indicate that LIFR may play an important role in the pathogenesis of corticotroph adenomas in the dog.

It can be concluded that it is unlikely that a mutated LIFR plays an important role in the pathogenesis of corticotroph adenomas in the dog. However, there is a strong co-expression of LIFR and POMC in the canine pituitary and activation of the LIFR pathway may still be important of the tumor formation.

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CHAPTER 10

The leukemia inhibitory factor receptor gene is not involved in the etiology of pituitary dwarfism in German Shepherd dogs

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Abstract

Pituitary dwarfism in German shepherd dogs is characterized by combined pituitary hormone deficiency (CPHD) and intrapituitary cyst formation. Activation of the leukemia inhibitory factor (LIF)-LIF Receptor (LIFR) signal transduction pathway results in a similar phenotype in (transgenic) mice. We therefore assessed the role of the LIFR in the etiology of pituitary dwarfism in German shepherd dogs. A polymorphic microsatellite marker (UULIFR) was used to analyze the segregation of the LIFR gene in 22 German shepherd dogs from 4 pedigrees, each including one dwarf. There was no allelic association between UULIFR and the dwarfism phenotype. Based on our findings LIFR was excluded as a candidate gene for CPHD.

In dogs, congenital growth hormone (GH) deficiency or pituitary dwarfism is the most striking example of pituitary hormone deficiency. The disorder is encountered most often as a simple, autosomal recessive inherited abnormality in German shepherd dogs (GSD).[3] The condition is characterized by profound dwarfism with retention of secondary (lanugo) hairs, and lack of primary (guard) hairs. German shepherd dwarfs have a combined deficiency of GH, prolactin (PRL), thyroid-stimulating hormone (TSH) together with impaired release of gonadotropins, whereas ACTH secretion is preserved.[8,9] The combined pituitary dwarfism is associated with intrapituitary cyst formation.[10]

The etiology of pituitary dwarfism in GSD is potentially a mutation of a receptor or a transcription factor that precludes effective expansion of a pituitary stem cell during or after the differentiation of the corticotrope cells. In humans and mice, combined deficiency of GH, TSH and PRL has been related to mutations in the genes encoding for DNA transcription factors Pit-1,[13,16] and Prophet of Pit-1 (Prop1).[7,17,19] However, DNA sequence and segregation analysis of Pit-1 and Prop-1 in GSD, revealed neither mutations nor co-segregation between the Pit-1 or Prop-1 alleles and the dwarfism phenotype,[11,12] and could thereby be excluded as candidate genes for pituitary dwarfism in GSD.

The LIM class homeodomain transcription factor LHX4 acts at an earlier stage of pituitary gland development. A splice-site mutation in the LHX4 gene has been identified in human patients with short stature, pituitary and hindbrain defects and abnormalities in the central skull base.[14] However, linkage analysis using a polymorphic DNA marker in the immediate vicinity of the canine LHX4 gene revealed no co-segregation of LHX4 allele and pituitary dwarfism in GSD.[18]

Leukemia inhibitory factor (LIF) is a pleiotropic cytokine that has an important role in the ontogeny of the adenohypophysis. When overexpressed in transgenic mice (driven by a pituitary-specific α-GSU promoter), it produces results similar to those seen in pituitary dwarfism in GSD: growth retardation, pituitary cysts outlined with a ciliated epithelium, and a diversion from the gonadotrope, thyrotrope, somatotrope, and lactotrope cell lineages to the corticotrope cell lineage.[20] This indicates that activation of the LIF-LIF receptor (LIFR) signal transduction pathway has a stimulatory role during the early development of the corticotrope lineage and may attenuate the other lineages. LIF transgenic mice exhibited hyperplasia of the pituitary corticotropes and Cushingoid feature[20] which are not seen in the GSD. The aim of this study was to investigate the role of LIF signaling pathway in the pathogenesis of pituitary dwarfism. It was hypothesized that possible constitutive activation of the LIFR or increased sensitivity of the LIFR towards LIF could play a role in the pathogenesis of pituitary dwarfism in GSD. Therefore, a polymorphic microsatellite marker in intron 4 of the canine LIFR was used to investigate the allelic association between LIFR locus and the dwarfism phenotype in GSD.

Materials and methods

Animals and DNA extraction

Twenty-two dogs from 4 litters that included one dwarf each were included in the study.[11,12,18] Blood samples were collected via jugular venipuncture, and genomic DNA was extracted by the salt extraction method of Miller *et al*. (1998).[15] Genomic DNA extracted from the spleen of a Great Dane was used as a control.

PCR amplification and sequence analysis of LIFR

The nucleotide DNA sequence of the canine LIFR gene was assembled from the boxer trace files whole genome sequencing project available at http://www.ncbi.nlm.nih.gov/Traces/-trace.cgi. The exon-intron organization of the canine LIFR gene was deduced by comparison with the human LIFR complementary DNA (cDNA) sequence (NM_002310). To verify the putative cDNA sequence, pituitaries were collected from two Labrador retrievers (1.5 and 2 years old), euthanized for reasons unrelated to pituitary abnormalities. The pituitaries were quick-frozen in liquid nitrogen and stored at -70º C until analysis. Total RNA was extracted using the RNeasy mini kit according to manufacturer's instructions (Quiagen, Venlo, The Netherlands). Complementary DNA was synthesized using oligo-dT primers and AMV Reverse Transcriptase according to manufacturer's instructions (Promega, Leiden, The Netherlands). RT-PCR was performed using 8 intron-spanning, overlapping primer-pairs covering the putative exon 2 to exon 21 sequences (Table 1). The PCR reactions were performed in a 50-µl volume containing 5 ng of genomic DNA, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of both primers, 2.5 U Recombinant Taq DNA polymerase (Invitrogen, Breda, The Netherlands) in PCR Reaction Buffer (Invitrogen, Breda, The Netherlands). The PCR program consisted of an initial activation at 94°C for 4 min followed by 35 cycles each of 1 min at 94°C, 1 min at 50-65°C, 1 min at 72°C, followed by a final extension at 72°C for 10 min. The products were visualized on ethidium-bromide containing 1.5% agarose gels. For DNA sequence analysis, the PCR products were diluted 1:10 in distilled water and 1 µl of the dilution was used in the sequence reaction, using the BigDye Terminator Cycle Sequencing Ready reaction (Applied Biosystems, Foster City, CA), according to the standard protocol. The tercycle reaction consisted of 25 cycles each of 30 sec at 96°C, 15 sec at 50°C or 53°C, and 2 min at 60°C. Tercycle products were purified using multiscreen 96-well filtration plates (Millipore, Amsterdam, The Netherlands), and analyzed on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). The LIFR cDNA sequence has been deposited in the GenBank database under Accession AY745241 / NM_001005760.

Table 1
Primers used to analyze the canine LIFR cDNA.

Exons	Forward primer	Reverse primer	T_a	Length
2-5	5'-CAAAGTTTCTGTGGCTGTGC-3'	5'-AAGATCTCTGGCGTGTCTGG-3'	65	524
3-6	5'-GCAGCAGGCCATGGTACTGT-3'	5'-GGCTCCAGTCACTCCACTCT-3'	65	505
6-9	5'-TTCATCACTGGAGTTGGACC-3'	5'-CCAGAGGATTGCGAGCATTC-3'	58	651
8-12	5'-ACTTGGAATCCAGGAAGACC-3'	5'-AAGGAACTGGCTTCTGTGGT-3'	50	554
11-14	5'-TCCGGATTCGCTGTTCTACT-3'	5'-TCCGACAGCCTGCTCTACTT-3'	50	395
13-17	5'-CACAAGGCGGAACTTCAACT-3'	5'-CCTCTGAGCTCTTCCACAGG-3'	50	488
16-19	5'-CGACCAGGTGTAAGATACAG-3'	5'-CCACAGGAATGAGAATGGCA-3'	58	460
18-21	5'-TACCATCTGGTCTTACGAGC-3'	5'-GAGGTGCATCTGTGGCTTGT-3'	50	603
19-21	5'-ATTCTCATTCCTGTGGCCGT-3'	5'-GCTCACTGAGATGGCAGACT-3'	53	817

T_a, Annealing temperature in degree Celsius; Length, PCR product length in base pair

Microsatellite marker in the LIFR gene

A $(CA)_{19}$ repeat marker was found in intron 4 (UULIFR) of the canine LIFR gene. The fragment of interest was amplified using primers flanking the CA dinucleotide-repeat and sequenced to confirm the identity of the fragment. The sequences of the primers used were 5'-ACTAGAGTTTTGTGGGAATC-3' and 5'-TGGTGGCTCAGGGTAGT-3'. The PCR

reactions were performed as described above at an annealing temperature of 55ºC. The 357 bp PCR product was diluted 1:5 in distilled water and 1 µl of the dilution was used in the sequencing reaction, as described above.

Genotyping of microsatellite markers

The 5'end of the forward primer was labeled with 6-FAM fluorescent dye (Eurogentec, Maastricht, The Netherlands). The PCR reactions were performed as described above. Labeled PCR products were diluted 1:10 and 2 µl of this dilution was mixed with 10 µl formamide and 0.2 µl of an internal size standard (GS-500 ROX, Applied Biosystem, Foster City, CA) for analysis on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). The data were analyzed with GENESCAN software 3.7 (Applied Biosystems, Foster City, CA). In one case of inconsistent inheritance, we analyzed segregation of the markers (REN214L11, FH2658, FH4060, FH3299, FH2869, FH2326, and FH3313). PCR reactions were performed in a 15-µl volume containing 25 ng of genomic DNA, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of both primers, 0.02 U AmpliTaq Gold (Applied Biosystem, Foster City, CA). The PCR program consisted of an initial activation at 95°C for 5 min, 10 cycles of 30 sec at 95°C, 15 sec at 55°C, 30 sec at 72°C, followed by 25 cycles of 30 sec at 92°C, 15 sec at 55°C, 30 sec at 72°C followed by a final extension at 72°C for 10 min. The PCR products were diluted 1:10 to 1:25 and processed and analyzed as described above, but with internal lane size standard GeneScan-500 LIZ (Applied Biosystem, Foster City, CA).

Results

Characterization of the canine LIFR cDNA

For each primer pair only one PCR product was identified on the agarose gel (Figure 1). DNA sequence analysis of the RT-PCR products confirmed the LIFR cDNA sequence. The deduced canine LIFR cDNA shared 87% similarities with human LIFR and was 78% homologous with murine LIFR. The amino acid sequence was 87% homologous with human LIFR and 75% homologous with the murine LIFR. The highest amino acid homology was seen in the carboxy terminus.

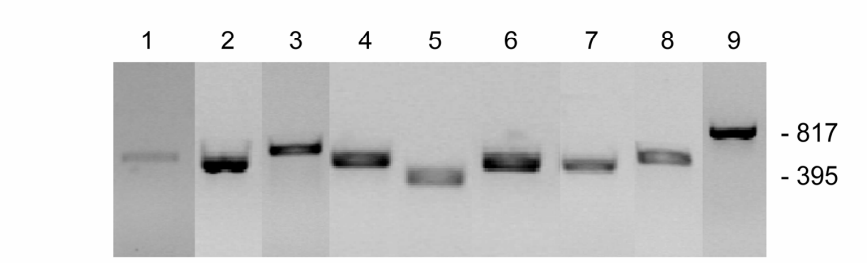

Figure 1. PCR of LIFR complementary DNA fragments synthesized from total RNA extracted from canine pituitary*, pars distalis adenohypophysis*. RT-PCR products visualized on ethidium-bromide containing 1.5% agarose gel. The bands represent products for each primer pair listed in Table 1; lane 1, exon 2-5; lane 2, exon 3-6; lane 3, exon 6-9; lane 4, exon 8-12; lane 5, exon 11-14; lane 6, 13-17; lane 7, exon 16-19; lane 8, exon 18-21; lane 9, exon 19-21.

Allelic association

The microsatellite marker in intron 4 of the LIFR gene was found to be polymorphic. The number of CA-repeats in the published boxer genome was 19, in the Great Dane control 14, which corresponded to the allelic length 357. In the GSDs two alleles were found: 363 that corresponded with 17 CA-repeats and 365 that corresponded with 18 CA-repeats. The genotypes of the 4 dwarfs were 363-363 (1 dog), 363-365 (1 dog), and 365-365 (2 dogs) (Figure 2). In the unaffected dogs the genotype was 363-363 (4 dogs), 363-365 (1 dog), and 365-365 (13 dogs) (Figure 2). The alleles segregated in 3 pedigrees (A, B, and D; Figure 2). In one pedigree (C; Figure 2) the LIFR alleles of two littermates did not correspond with those of the parents, but 7 other microsatellite markers were highly informative and segregated. Hence, the pedigree was considered consistent.

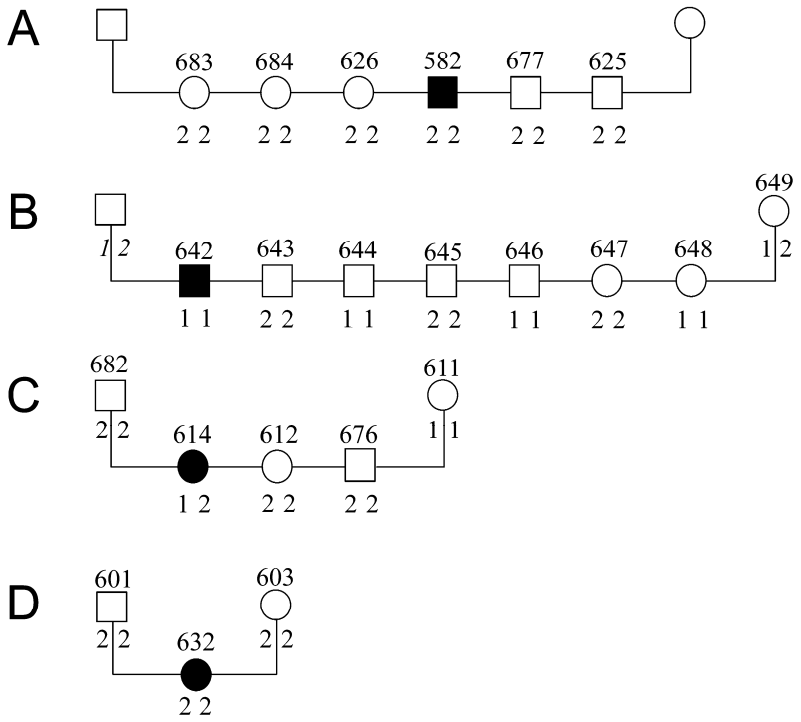

Figure 2. The segregation of the leukemia inhibitory factor receptor microsatellite in 4 German shepherd dog pedigrees. The dwarfism phenotype is indicated with closed symbols, squares: males, circles: females. Symbols without a number represent animals which were not available for analysis. The LIFR CA-repeat alleles are shown below the symbols. Allele 1: 363 bp fragment, allele 2: 365 bp fragment. The genotype of the sire in pedigree B was deduced from the littermates (italic).

Discussion

Leukemia inhibitory factor (LIF), a protein belonging to the IL-6 cytokine family, and its receptor (LIFR), play an important modifying role in the mature hypothalamo-pituitary-adrenal axis as well as in the ontogeny of the adenohypophysis.[4] The presence of both LIF and LIFR have been demonstrated in the pituitary of mice and humans.[2,4] The results of the present study show that LIFR is also expressed in the canine pituitary. The canine LIFR mRNA encodes a protein of 1097 amino acids. The carboxy terminal part, which has an intracellular localization, appears to be the region with the highest level of conservation among species.

In the linkage analysis, using the UULIFR microsatellite, there was an unexplainable inheritance of alleles in pedigree C. Therefore, the accuracy of the pedigree information was checked with 7 microsatellite markers from other locations of the genome, which were highly informative, and segregated in the pedigree. The aberrant finding in the UULIFR marker could be explained by a mutation of the CA-repeat from one copy of allele 1 to allele 2, or presence of a null allele in the mother´s genome. A mutation in the number of repeat sequences is thought to arise through replication slippage by the Taq polymerase, but there are conflicting data presented over the mutation rate in CA-repeats in mammalian cells.[6] There are many possible causes of a non-amplifiable allele,[5] but the most likely explanation for the findings in pedigree C is a mutation in one of the two primer binding sites. This was not investigated further because the findings still suffice to exclude the LIFR as candidate gene. Because the inheritance of the dwarfism phenotype is recessive,[3] it can be expected that all dwarfs have the same genotype, and that they would be homozygous for the same defect allele. However, the four dwarfs in our study showed three different genotypes for the UULIFR marker.

German shepherd dogs (GSD) with pituitary dwarfism have hypofunctioning somatotropes, gonadotropes, lactotropes, and thyrotropes, but normal function of corticotropes,[9] and, commonly, also cystic enlargements in the adenohypophysis. These features were also seen in transgenic mice with pituitary overexpression of LIF under a pituitary-specific α-GSU promoter, starting at embryonic day 9.5 which is before the differentiation of the corticotrope lineage (e11.5).[20] In a GH promoter-driven LIF transgenic mouse in which the overexpression of LIF starts later during embryonic development (e17.5), similar impairment of the development of GH secreting cells and formation of pituitary cysts have been demonstrated.[1] Based on these observations it was hypothesized that a mutation in the canine LIFR, resulting in constitutive activation of the LIF-LIFR signal transduction pathway, could be involved in the etiology of pituitary dwarfism in the GSD. However, the results of this study indicate that a mutation of LIFR can be ruled out. Due to the possible presence of null-alleles we cannot use the marker for classical linkage approach. However, we can maintain the conclusion that the dwarfs have different genotypes. Chance of recombination is negligible, as the UULIFR marker is situated within the gene of interest. Although we have excluded mutations in LIFR as the cause of GSD dwarfism, it cannot be excluded that either local pituitary overexpression of LIF, or downstream effectors of the LIF-LIFR signal transduction pathway are involved in the pathogenesis of pituitary dwarfism in GSD. The search for the genetic cause of CPHD in the German shepherd dog remains to be elucidated.

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CHAPTER 11

Summarizing discussion and conclusions

Summarizing discussion

The pituitary gland is a small structure attached to the underside of the brain. The pituitary gland rests in the pituitary fossa of the sphenoid bone, which separates the nasal and cranial cavities **(Chapter 2)**. Despite its modest size, the pituitary gland regulates vital body functions such as stress reaction, growth, lactation, basal metabolism, reproduction, and water retention.[9]

Pituitary-dependent hyperadrenocorticism (PDH) is a common endocrinopathy in the elderly dog caused by an ACTH-secreting (corticotroph) pituitary adenoma[8] with unknown molecular pathogenesis. The dogs suffer from typical signs of hypercortisolism and sometimes also from tumor mass effects.[9] The most commonly used medical therapies (mitotane and trilostane) target the adrenal glands to reduce cortisol production.[6] To address the primary cause, that is the pituitary adenoma, a technique for surgical removal of the pituitary gland has been developed and was applied as treatment of dogs with PDH in the Netherlands since 1993. The first results were promising in the treatment of PDH in dogs using this approach.[4,5]

There were two major aims of this thesis **(Chapter 1)**: Firstly, to analyze the long-term results for transsphenoidal hypophysectomy and investigate whether there are predictors for surgical outcome, and secondly, to reveal the underlying pathogenesis by molecular biological studies of the pituitary adenomas.

The 10-year follow-up results in 150 dogs with PHD confirmed that transsphenoidal hypophysectomy, in the hands of a skilled neurosurgeon, is an effective treatment for PDH in dogs, especially on the long term, with dogs in remission for up to seven years after surgery **(Chapter 3)**. However, with surgical treatment there are complications, e.g., postoperative mortality, residual disease, recurrences, central diabetes insipidus, and keratoconjunctivitis sicca. In **Chapter 3** it was concluded that dogs with PDH and enlarged pituitary glands, following hypophysectomy, had shorter survival and disease-free periods and an increased risk of permanent central diabetes insipidus.

With regression analysis performed on preoperative variables in a group of 181 dogs with PHD it was possible to identify predictive variables for survival and recurrences after transsphenoidal hypophysectomy **(Chapter 4)**. For example, older dogs and dogs with high plasma concentration of ACTH have higher risk of PHD-related mortality. Large pituitary size, relatively thick sphenoid bone, high preoperative UCCR, and high plasma α-MSH concentrations are associated with increased risk of recurrence. As a direct result of this study, the introduction of the use of a rigid endoscope after pituitary tumor extraction was motivated in an attempt to reduce the increased risk of recurrences in dogs with a thick sphenoid bone that limits the surgical exposure.

Postoperatively, there may be remnant functional pituitary corticotroph cells, even after hypophysectomy of a normal pituitary gland. For hypophysectomized dogs with PDH, the question arises whether it is possible to differentiate between remnant normal and adenomatous pituitary tissue. In the study on pulsatile variation in hormone concentrations in 17 dogs with PDH, before and after hypophysectomy **(Chapter 5)**, the presence of pulsatile variation in ACTH secretion postoperatively was predictive for recurrence. However, measurement of the 6-hour variations in plasma hormone concentrations remains a research tool and is not practical for routine use.

In a more practical set-up, the perioperative hormone plasma profiles of ACTH, cortisol, α-MSH and GH in 51 dogs with PDH were evaluated as predictors for surgical outcome. Indeed, blood sampling for ACTH, cortisol, and α-MSH after surgery proved to be a helpful and easy-to-use tool in predicting the long-term outcome after transsphenoidal hypophysectomy **(Chapter 6)**.

It can be concluded that these studies have contributed valuable information about the efficacy of transsphenoidal hypophysectomy as treatment of PDH in dogs. Initial remission is seen in 85% of the dogs, but signs of hyperadrenocorticism will recur in 25% of these dogs, up to 4 years after hypophysectomy. Preoperative measurement of the plasma α-MSH and ACTH concentration, the UCCR, and pituitary imaging give prognostic information about the surgical outcome. Measurement of the immediate postoperative plasma α-MSH, ACTH and cortisol and 8-week postoperative measurement of UCCR are useful to evaluate the actual surgical outcome. However, it is not possible to predict every single recurrence. It is, therefore, important to conduct regular postoperative controls in these patients. To further improve the surgical outcome, further investigations on preoperative and postoperative evaluation and treatment would be of interest.

The second part of this thesis is focused on factors (Tbx19/Tpit, NeuroD1, LIF-LIFR) that promote the normal differentiation of corticotroph cells during pituitary organogenesis.[3,7,11] In the pituitary gland, the Tbx19/Tpit is specific for the ACTH and α-MSH producing cells.[3] The complete coding sequence of the canine Tbx19 was sequenced and a mutation analysis performed on cDNA derived from tissue specimens of 14 corticotroph adenomas. No tumor-specific mutation was found, but interestingly, a missense polymorphism was discovered in the most conserved part of the protein (the Tbox) of a Bernese Mountain dog. With extended investigation in a control group of Bernese Mountain dogs, an allele frequency of 25% was determined. The influence on the function of Tbx19 is unknown **(Chapter 7)**.

NeuroD1 is a transcription factor that is expressed in the corticotrophs, but not in the melanotrophs, in mice. With quantitative-PCR analysis it was confirmed that NeuroD1 is mainly expressed in the canine *pars distalis adenohypophysis* and that the expression is low in neurointermediate lobe **(Chapter 8)**. NeuroD1 was differentially expressed in the corticotroph adenomas and may become useful for the division of the adenomas into subtypes.

The coding sequence of the canine LIFR was sequenced, and cDNA from 14 corticotroph adenomas were screened but revealed no mutations. However, there was a strong co-expression of LIFR and POMC and the expression of LIF was surprisingly low in the *pars intermedia adenohypophysis*. Our findings together with those made by others,[1] are indicative that the LIFR is an important factor for the pathogenesis of corticotroph adenomas in the dog, and that LIFR may serve a non-neuronal regulatory mechanism for hormone secretion in the *pars intermedia adenohypophysis*. The latter finding becomes highly interesting with respect to the recently recognized anti-inflammatory properties of α-MSH.[2,10] In future studies it would be interesting to investigate the influence of cytokines on the secreting activity of the canine *pars intermedia adenohypophysis*. It may be hypothesized for future research that chronic cytokine stimulation may participate in the pathogenesis of PDH in the dog **(Chapter 9)**. LIFR was excluded as a causative factor of pituitary dwarfism in German Shepherd dogs **(Chapter 10)**.

It can be concluded from the studies in the second part of this thesis, that Tbx19/Tpit is a useful marker for ACTH and α-MSH producing cells in the pituitary. These cells also have a high expression of the LIFR, but the expression of NeuroD1 is different. Very interesting for further investigations is the possible presence of other cytokines in the pituitary and the direct effects of those on hormonal secretion at the pituitary level, especially their direct effects on α-MSH secretion from the intermediate lobe. Also continued studies would be of interest to investigate the possible role of NeuroD1 as a marker for tumors originating from the *pars distalis adenohypophysis*.

Conclusions

Part I Pathobiology of pituitary function after transsphenoidal hypophysectomy in dogs with pituitary-dependent hyperadrenocorticism

► Transsphenoidal hypophysectomy is an effective treatment for pituitary-dependent hyperadrenocorticism in dogs.
► Survival and disease-free fractions after hypophysectomy decrease and the incidence of CDI increases with increasing pituitary size.
► Pituitary size, thickness of the sphenoid bone, plasma α-MSH concentration and urinary cortisol excretion before hypophysectomy are predictors of long-term remission.
► Presence of ACTH pulses in plasma hormone concentration after hypophysectomy is a risk factor for recurrence of hyperadrenocorticism.
► Peri-operative plasma profile of adrenocorticotrophic hormone predicts recurrence of hyperadrenocorticism after hypophysectomy.

Part II Oncogenesis of corticotroph pituitary adenoma in dogs

► Pituitary T-box transcription factor Tbx19/Tpit can be used as a reliable marker for corticotroph and melanotroph cells in canine pituitary tissue and mutations in the Tbx19/Tpit gene are unlikely to play a major role in the pathogenesis of canine corticotroph adenomas.
► Neurogenic differentiation 1 (NeuroD1) is mainly expressed in the cells of *pars distalis adenohypophysis*, the expression of NeuroD1 in the neurointermediate lobe is low.
► LIFR is strongly co-expressed with POMC in the canine pituitary gland and the expression of LIF is low in the *pars intermedia adenohypophysis*. Our findings together with those made by others, are indicative that the LIFR is an important factor for the pathogenesis of corticotroph adenomas in the dog.
► LIFR is not involved in the pathogenesis of combined anterior pituitary deficiency in German Shepherd dogs.

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Samenvatting

Bij de oudere hond is hypofyse-afhankelijk hyperadrenocorticisme (HAH) of de ziekte van Cushing, een vaak voorkomende endocriene aandoening. HAH wordt veroorzaakt door een adrenocorticotropine (ACTH) producerende tumor (corticotroop adenoom) in de hypofyse, een aanhangsel onder aan de hersenen.[9] ACTH stimuleert de synthese van cortisol in de bijnierschors en de secretie van dit hormoon. Als gevolg hiervan krijgen honden met HAH verschijnselen van hypercortisolisme, zoals veel urineren (polyurie), veel drinken (polydipsie), vraatzucht (polyfagie), een dikke buik, dunne vacht en huid, spieratrofie en een verminderd uithoudingsvermogen.[8] Neurologische verschijnselen worden veroorzaakt door een centraal tumor massa effect. De meest gebruikte medicamenteuze behandelingen (mitotane en trilostane) werken op het niveau van de bijnierschors en remmen daar de cortisol productie.[5,6] De primaire oorzaak, het hypofyse adenoom, blijft dan onbehandeld. Sinds 1993 worden honden causaal behandeld door middel van transsphenoidale hypofysectomie aan de Faculteit Diergeneeskunde van de Universiteit te Utrecht.[3,4] Het is onbekend hoe de hypofyse adenomen ontstaan **(Hoofdstuk 2)**.

In dit proefschrift werden de lange termijn resultaten onderzocht van honden die een hypofysectomie hebben ondergaan in relatie tot overleving, recidive en prognostische factoren. Tevens werd het moleculair biologische karakter van de tumoren bestudeerd **(Hoofdstuk 1)**.

De resultaten van 10 jaar follow-up studie van 150 geopereerde honden met HAH bevestigen dat hypofysectomie, door een ervaren chirurg, een effectieve behandeling is op de lange termijn **(Hoofdstuk 3)**. De complicaties die kunnen ontstaan zijn postoperatieve mortaliteit, onvolledige remissie, recidive van de klachten, keratoconjunctivitis sicca en polydipsie veroorzaakt door een centrale diabetes insipidus. Honden met HAH en een vergrote hypofyse die geopereerd werden hebben een kortere overlevingstijd, een hogere kans op recidive en ook een hoger risico om een centrale diabetes insipidus te ontwikkelen, dan honden met een niet vergrote hypofyse.

Regressie-analyse van preoperatieve variabelen van 181 geopereerde honden leverde een aantal factoren op die een voorspellende waarde hadden voor de uitkomst na de operatie. Kortere overlevingstijd werd gezien bij oudere honden en honden met een hoge plasma ACTH concentratie. Risicofactoren voor recidive waren een vergroting van de hypofyse, een dik sphenoid, een hoge preoperatieve corticoïd/creatinine verhouding in de urine (UCCR) en een hoge plasma concentratie van α-melanocyt-stimulerend hormoon (α-MSH).

Na hypofysectomie blijven vaak microscopische eilanden van functionerende corticotrope cellen achter. Bij honden met HAH zou het daarom interessant zijn om onderscheid te kunnen maken tussen restanten normale en tumoreuze cellen. Hiervoor werd de pulsatiele variatie in plasma concentraties van ACTH, cortisol, α-MSH en GH vóór en 6 tot 8 weken na hypofysectomie geanalyseerd. Uit dit onderzoek bleek dat een pulsatiele variatie in de plasma ACTH concentratie in verband staat met een verhoogd risico van recidive van hyperadrenocorticisme **(Hoofdstuk 5)**.

Omdat bepaling van de pulsatiele variaties in plasma hormoon concentraties arbeidsintensief en duur is, werd een meer praktisch protocol ontwikkeld waarbij plasma hormoon concentraties van ACTH, cortisol, α-MSH en GH tijdens de eerste 4 uur na de operatie werden bepaald. Uit deze studie bleek dat de preoperatieve plasma α-MSH concentratie en

postoperatieve concentratie van plasma ACTH, cortisol en α-MSH factoren met een voorspellende waarde waren voor recidive van hyperadrenocorticisme **(Hoofdstuk 6)**.

Bovenstaande studies hebben belangrijke informatie opgeleverd over de effectiviteit van transsphenoidale hypofysectomie als behandeling van HAH bij de hond. Circa 85% van de honden zijn initieel in remissie gegaan. In ongeveer 25% van deze honden komt de ziekte terug. Met de preoperatieve bepaling van de plasma α-MSH en ACTH concentratie en de UCCR, alsmede met kennis van de grootte van de hypofyse, is het mogelijk om prognostische informatie te geven over het te verwachten resultaat. Een direct postoperatieve analyse van de plasma α-MSH, ACTH en cortisol concentratie en de bepaling van de UCCR op 8 weken na de operatie zijn belangrijke waarden in de voorspelling van de chirurgische uitkomst op de lange termijn. Het is echter niet mogelijk om iedere recidive te voorspellen. Een regelmatige controle van de geopereerde honden is daarom van belang. Voor de toekomst zullen verdere studies van pre- en postoperatieve klinische beoordeling en behandeling interessant zijn. Naar aanleiding van het onderzoek in deel I werd een intraoperatieve endoscopische controle geïntroduceerd na hypofysectomie om eventuele hypofyse restanten op te sporen en daarmee het recidieve percentage te verminderen.

In het tweede deel van dit proefschrift werd de rol van factoren (Tbx19/Tpit, NeuroD1, LIF-LIF-receptor) onderzocht die de differentiatie stimuleren van ACTH en α-MSH producerende cellen tijdens de embryonale ontwikkeling van de hypofyse.[2,7,11] De Tbx19/Tpit factor is een specifieke factor voor ACTH en α-MSH producerende cellen in de hypofyse.[2] De coderende sequentie van het Tbx19 cDNA werd vastgesteld en een mutatieanalyse werd gedaan op weefsel van 14 ACTH producerende adenomen. Geen tumorspecifieke mutatie werd gevonden. Interessant genoeg werd een missense polymorfisme gevonden in een geconserveerd gebied van het DNA bindend domein (T-box). De allel frequentie van dit polymorfisme bij Berner Sennen honden was 25%, hoewel het niet bekend is welke invloed deze mutatie op de functie van de Tbx 19 heeft **(Hoofdstuk 7)**.

NeuroD1 komt niet tot expressie in de α-MSH producerende cellen van de muis. Dit werd ook bevestigd met kwantitatieve PCR (qPCR) bij de hond. De expressie van NeuroD1 in de *pars distalis adenohypophysis* was significant hoger dan de expressie in de neurointermediaire laag **(Hoofdstuk 8)**. De expressie van NeuroD1 van de ACTH producerende adenomen was verschillend. Dit kan misschien in de toekomst gebruikt worden om de adenomen in groepen te verdelen.

Een sequentie- en mutatieanalyse van de coderende sequentie van LIFR bij de hond toonde geen mutaties. Daarentegen was er een uitgebreide co-expressie van LIFR, ACTH en α-MSH **(Hoofdstuk 9)**. Activatie van LIFR zou hypothetisch betrokken kunnen zijn in de pathogenese van HAH bij de hond. Ondanks de hoge expressie van de LIFR was de expressie van LIF opvallend laag, vooral in de intermediaire laag. Deze bevinding kan erop duiden dat LIFR een niet-neurale mediator is voor stimulatie van α-MSH secretie uit de intermediaire laag. Gezien de ontstekingsremmende eigenschappen van α-MSH[1,10] is deze bevinding bijzonder interessant. LIFR werd ook uitgesloten als causale factor van hypofysaire dwerggroei bij de Duitse Herder **(Hoofdstuk 10)**.

Het onderzoek in deel II van dit proefschrift toonde aan dat de Tbx19/Tpit de ACTH en α-MSH producerende cellen kan identificeren. Deze cellen hebben ook een hoge expressie van LIFR, maar de expressie van NeuroD1 is verschillend. Voortgezet onderzoek kan bestaan uit studies naar de rol van ontstekingsmoleculen in de regulatie van de hormoon-

secretie in de hypofyse, in het bijzonder in de intermediaire laag. Ook zou het interessant zijn om de rol van NeuroD1 te bestuderen als marker voor corticotrope adenomen.

Conclusies

Deel I. De pathobiologie van de hypofyse-functie na transsphenoidale hypofysectomie bij honden met hypofyse-afhankelijk hyperadrenocorticisme bij de hond.

► Transsphenoidale hypofysectomie is een effectieve behandeling voor hypofyse-afhankelijk hyperadrenocorticisme.
► Naarmate de hypofysetumor groter is zal, na transsphenoidale hypofysectomie, de overlevingstijd korter en het recidive percentage hoger zijn, en de kans op (permanente) diabetes insipidus toenemen.
► De grootte van de hypofyse, de dikte van het sphenoid, de plasma α-MSH concentratie en de UCCR vóór de operatie, zijn prognostische factoren voor recidive na de operatie.
► Aanwezigheid van een meetbare pulsatiele secretie van ACTH na hypofysectomie is een risicofactor voor recidive van hyperadrenocorticisme op termijn.
► Analyse van het plasmaprofiel van ACTH direct na de operatie helpt de lange termijn uitkomst met betrekking tot recidive te voorspellen.

Deel II . Oncogenese van het corticotrope hypofyse adenoom bij de hond.

► Tbx19/Tpit is een marker van ACTH en α-MSH producerende cellen bij de hond. Het is niet waarschijnlijk dat mutaties in Tbx19/Tpit een doorslaggevend belang hebben in de ontwikkeling van het ACTH producerend adenoom bij de hond.
► NeuroD1 komt vooral tot expressie in de *pars distalis adenohypophysis* en de expressie is laag in de intermediaire laag.
► Er is een opvallende co-expressie van de leukemia inhibitory factor receptor (LIFR), ACTH en α-MSH in de hypofyse van honden. Onze bevindingen en die van andere groepen, wijzen op een belangrijke rol voor LIFR in de pathogenese van het corticotrope adenoom bij de hond.
► De receptor voor LIF (LIFR) is niet betrokken in de pathogenese van hypofysaire dwerggroei bij de Duitse Herder.

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Sammanfattning

Hypofysberoende hyperadrenokorticism (HHA), eller Cushings sjukdom, är en vanlig sjukdom hos medelålders till äldre hundar, som orsakas av en adrenokortikotropt hormon (ACTH)-producerande tumör (kortikotropt adenom) i hypofysen (nedre hjärnbihanget).[9] ACTH verkar på binjurarna, vilka stimuleras till en ökad produktion och utsöndring av stresshormonet kortisol. Därför uppvisar hundar med HHA kliniska tecken på hyperkortisolism, till exempel ökad urinavgång (polyuri), ökad törst (polydipsi), ökad aptit (polyfagi), ökat bukomfång, håravfall, tunn och skör hud, muskelförtvining och nedsatt uthållighet. Dessutom kan hundarna uppvisa centralnervösa symtom orsakade av en direkt tryckeffekt från hypofystumören.[2,9] De vanligaste medicinska behandlingarna (dvs mitotan och trilostan) utövar sina effekter på binjurarna,[6,7] där de minskar kortisolproduktionen. Den primära orsaken, dvs. hypofystumören kvarstår dock. Med syfte att rikta behandlingen mot hypofystumören har en kirurgisk teknik (transsfenoidal hypofysektomi) tillämpats i Nederländerna sedan 1993.[4,5] Det är inte känt, vad som orsakar kortikotropa adenom, inte heller vet man varför det är så vanligt förekommande hos hund **(Kapitel 2)**.

Studierna i denna avhandling har haft två övergripande mål **(Kapitel 1)**; (1) att göra en uppföljande långtidsstudie av hundar som genomgått hypofysektomi vad gäller överlevnad, återfall och prognostiska faktorer; (2) att utföra molekylärbiologiska studier i ett försök att klarlägga de kortikotropa adenomens patogenes.

Resultatet från en uppföljande studie av 150 opererade hundar med HHA, som sträckte sig över 10 år, konfirmerade att hypofysektomi är en effektiv behandlingsmetod i händerna på en skicklig neurokirurg **(Kapitel 3).** Till komplikationerna hör postoperativ mortalitet, persisterande hyperadrenokorticism, återfall, central diabetes insipidus och keratokonjunktivitis sicca. Hundar med HHA och en förstorad hypofys har kortare livslängd, kortare återfallsfri period, samt löper större risk att utveckla persisterande central diabetes mellitus.

Med regressionsanalys av preoperativt tillgängliga parametrar för 181 behandlade hundar, kunde prediktiva faktorer för överlevnad och återfall identifieras **(Kapitel 4)**. Till exempel löper äldre hundar och hundar med hög plasma koncentration av ACTH en högre risk för HHA-relaterad mortalitet. En förstorad hypofys, tjockt sfenoidben, hög preoperativ urin kortikoid/kreatinin ratio (UCCR) och hög plasma koncentration av α-melanocyt-stimulerande hormon (α -MSH) är förenat med en ökad återfallsfrekvens.

Efter hypofysektomi finns det ofta mikroskopiska öar av kvarsittande funktionerande kortikotropa celler. Hos hundar med HHA är det av intresse att kunna differentiera mellan kvarsittande normala och tumörformade celler. I en studie av pulsativ variation i plasmakoncentrationerna av ACTH, kortisol, α-MSH och GH före och efter hypofysektomi var 6-8 veckor postoperativ pulsativ variation av plasma ACTH-koncentration förenad med ökad riks för återfall **(Kapitel 5)**.

Studier av pulsativa variationer av plasma koncentrationer av hormon är både arbetskrävande och kostsamt. I en mer praktiskt uppsättning analyserades värdet av en direkt mätning av plasmakoncentration av ACTH, kortisol, α -MSH och GH under de 4 första timmarna efter operationen hos 51 opererade hundar med HHA. Preoperativ plasma α-MSH koncentration och postoperativ plasma ACTH, kortisol och α -MSH var prediktiva för återfall (**Kapitel 6**).

Sammanfattningsvis studierna i avhandlingens första del bidragit med värdefull information om effektiviteten av transsfenoidal hypofysektomi som behandling av HHA hos

hund. Cirka 85% av hundarna blir initialt botade. Dock återkommer symtom på hyperadrenokorticism i 25% av fallen. Preoperativ mätning av plasma α-MSH och ACTH koncentration och UCCR samt visualisering av hypofysen och omgivande strukturer kan ge prognostisk information om det kirurgiska utfallet, i fråga om postoperativa komplikationer och återfallsrisk. Direkt postoperativ analys av plasma koncentration av α-MSH, ACTH och kortisol samt analys av UCCR 8 veckor efter operation är användbara parametrar för att utvärdera det kirurgiska utfallet också på längre sikt. Det är dock inte möjligt att förutspå samtliga återfall. Därför är genomförandet av regelbundna postoperativa kontroller av stor betydelse. Även fortsatta studier av pre- och postoperativa kliniska utvärderingar och behandlingar är av intresse för att ytterligare förbättra behandlingsresultaten. Som en direkt följd av dessa resultat har en intraoperativ endoskopisk efterkontroll introducerats för att minska återfallsfrekvensen hos hundar med ett tjockt sfenoid ben.

I avhandlingens andra del fokuseras faktorer (Tbx19-Tpit, NeuroD1, LIF-LIFR), som främjar den normala differentieringen av kortikotropa celler under fosterutvecklingen[3,8,11]. I hypofysen är Tbx19 specifik för ACTH och α-MSH producerande celler.[3] Den kompletta, koderande cDNA-sekvensen på hund fastställdes och en mutationsanalys genomfördes på tumörmaterial från 14 kortikotropa adenom. Ingen tumörspecifik mutation hittades. Intressant nog upptäcktes en missens polymorfism i en mycket välkonserverad DNA-bindande domän (T-boxen), med en allelfrekvens på 25% inom Berner Sennen hundar. Hur denna polymorfism påverkar Tbx19s funktion är okänt **(Kapitel 7**).

NeuroD1 är inte utrryckt i melanotropa celler hos mus. Detta fynd konfirmerades hos hund med kvantitativ qPCR analys. Uttrycket av NeuroD1 i *pars distalis adenohypofysis* och var signifikant högre än det i neurointermediärloben **(Kapitel 8)**. Det var en skillnad mellan NeuroD1s uttryck mellan olika kortikotropa adenom, vilket kan komma att bli användbart för att dela in dem i olika subgrupper.

En sekvens- och mutationsanalys av den koderande sekvensen av LIFR hos hund genomfördes, men inte heller här påträffades några mutationer. Däremot kunde en stark coexpression av LIFR och ACTH samt α-MSH påvisas **(Kapitel 9)**. Kronisk aktivering av LIFR skulle hypotetiskt kunna vara involverad in patogenesen av HHA hos hund. Trots det starka uttrycket av LIFR i intermediärloben, var uttrycket av LIF förvånande lågt. Detta fynd indikerar att LIFR kan mediera en icke-neural signaleringsväg för stimulering av α-MSH sekretion från intermediärloben. Detta är särskilt intressant med tanke på α-MSHs, på senare år uppmärksammade, direkta antiinflammatoriska verkan.[1,10] LIFR var utesluten som orsakande faktor för hypofysär dvärgväxt hos Schäfer **(Kapitel 10)**.

Sammanfattningsvis har studierna i avhandlingens andra del bekräftat användandet av Tbx19/Tpit som en markör för ACTH- och α-MSH producerande celler hos hund. Dessa celler uppvisar också starkt uttryck av LIFR men uttrycket av NeuroD1 är olika. Fortsatta studier av cytokinförekomst och dess reglering av hormonsekretionen i hypofysen och i intermediärloben i synnerhet, skulle vara mycket intressant, liksom studier av NeuroD1 som möjlig markör för tumörer härrörande från kortikotropa celler.

Konklusioner

Del I1 Patobiologi av hypofysfunktion efter transfenoidal hypofysektomi på hundar med hypofysberoende hyperadrenokorticism

►Transsfenoidal hypofysektomi är en effektiv behandling för hypofysberoende hyperadrenokorticism hos hund
►Överlevnad och återfallsfri period efter hypofysektomi minskar och incidensen av central diabetes insipidus ökar med ökad hypofysstorlek
►Hypofysens storlek, sfenoidbenets tjocklek, plasma α-MSH koncentration och kortisolexkretion i urinen preoperativt fungerar som prediktiva faktorer för återfall efter operation
► Förekomst av postoperativa pulsativa förändringar av plasma ACTH koncentrationen efter hypofysektomi är en riskfaktor för återfall av HHA.
► Tidig postoperativ analys av plasmaprofiler av ACTH kan förutsäga återfall av hyperadrenokortisism efter hypofysektomi

Del II Onkogenes av kortikotropa hypofysadenom hos hund

► Tbx 19-Tpit kan användas som en markör för ACTH och α-MSH producerande celler hos hund. Det är inte troligt att mutationer i Tbx19-Tpit är av avgörande betydelse i de kortikotropa adenomens patogenes
►NeuroD1 är i huvudsak utryckt i pars distalis adenohypophysis och uttrycket är lågt i intermediärloben.
► Det råder en stark coexpression av LIFR och ACTH och α-MSH i hundens hypofys. Våra fynd tillsammans med fynd gjorda av andra, indikerar att LIFR kan vara en viktig faktor för kortikotropa adenom hos hund.
► LIFR är inte involverad i patogenesen av hypofysär dvärgväxt hos Schäfer.

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Curriculum vitae

De schrijfster van dit proefschrift, werd op 14 juni 1970 geboren in Örebro te Zweden. Na het behalen van het VWO-diploma, richting Natuurwetenschap (met maximale cijfers; 5.0/5.0) aan de Karolinska Skolan te Örebro, Zweden, studeerde en werkte zij gedurende 2 jaar. In september 1991 ving zij aan de studie Diergeneeskunde te Uppsala, Zweden. Tussen september 1991 en december 1996 schreef ze 2 peer-reviewed artikelen en voltooide zij haar opleiding tot dierenarts. Tussen 1997 en 2002 was ze werkzaam als dierenarts, bij het dieren-ziekenhuis Falu Djursjukhus te Falun, Zweden. Tussen 1997-2000 werd de opleiding tot Zweedse specialist in de ziekten van honden en katten doorlopen. In 2000, werd met goed gevolg het examen afgelegden werd zij erkend als Zweedse specialist in de ziekten van de hond en de kat door Sveriges Veterinärmedicinska Sällskap (SvS) en Statens Jordbruksverk (Swedish Board of Agriculture). In september 2003 begon de promovenda haar opleiding bij het Departement Geneeskunde van Gezelschapsdieren, Faculteit Diergeneeskunde, Utrecht Universiteit. De promovenda is in 1995 getrouwd met Johannes Hanson en in 2000 en 2002 moeder geworden van Simon en Jakob.

The author of this thesis was born in Örebro, Sweden, on June 14th 1970. After graduating from secondary school 'Naturvetenskaplig linje' at Karolinska Skolan in Örebro, Sweden, she studied and worked for 2 years. In 1991, she started her veterinary studies at the Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences. During this time (September 1991-December 1996), she wrote two peer-reviewed articles and graduated as a veterinarian. During 1997 and 2002 the author worked as veterinarian at the Falu animal hospital, Falun, Sweden. During 1997-2000 she followed a 3-year specialization program and in 2000 she became "Swedish specialist in diseases of dogs and cats". In September 2003, she started her PhD studies at the Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, The Netherlands. In 1995, the author married Johannes Hanson and in 2000 and 2002, she became mother of Simon and Jakob.

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