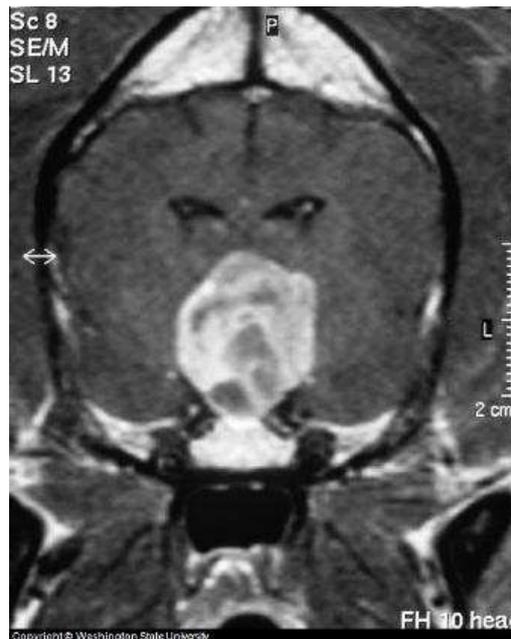


Expression of Pax7 and Sox2 in pituitary corticotroph adenomas in dogs

Master Thesis

Faculty of Veterinary Medicine

Utrecht University



Canine Pituitary Tumor in dog with Cushing's Disease
Source: Washington State University

Student: Joanne van der Bend

Student number: 3546152

Supervisor: Drs. Sarah van Rijn

Period: 11-03-2013 - 07-06-2013

Contents

Abstract	3
Introduction	
<i>Cushing's disease</i>	4
<i>Diagnosis of Cushing's disease</i>	5
<i>Treatment of Cushing's disease</i>	5
<i>The role of pituitary stem cells in pituitary tumorigenesis</i>	6
<i>The role of Pax7 in pituitary tumors</i>	7
<i>The role of Sox2 in pituitary tumors</i>	7
<i>Pax7+/Sox2+ cell population(s)</i>	8
<i>The aim of this study</i>	9
Materials & Methods	
<i>Animals</i>	9
<i>Histology and immunohistochemistry</i>	9
<i>Optimization of immunohistochemistry protocols</i>	11
<i>IHC for Pax7 and Sox2</i>	12
<i>Analysis of Pax7- and Sox2-expression</i>	12
<i>Statistical analysis</i>	13
Results	
<i>Description of Pax7- and Sox2-expression</i>	13
<i>Quantification of Pax7- and Sox2-expression</i>	15
Discussion	21
References	24
Appendix 1	26

Abstract

Cushing's disease, or pituitary dependent hypercortisolism (PDH), is a common endocrine disease in dogs, caused by a pituitary tumor that produces an excess of the hormone ACTH, which in turn leads to an excess of the hormone cortisol, produced by the adrenal gland.

Surgical removal of the pituitary gland by transsphenoidal hypophysectomy has become an important treatment method of Cushing's disease; recurrence rates turn out to be lower and dogs survive longer than dogs treated with drugs such as Mitotane or Trilostane. The prognosis also depends on the size of the pituitary. Enlarged pituitaries containing a macroadenoma have been shown to have a worse prognosis than non-enlarged pituitaries containing a microadenoma.

In search for possible pituitary stem-/progenitor cell populations that may play a role in pituitary tumorigenesis, some progenitor-markers have been identified, among which Pax7 and Sox2. In this study, the expression of transcription factors Pax7 and Sox2 was examined in different canine pituitary (tumor) tissues, by means of immunohistochemistry; microadenomas, macroadenomas, malignant tumors, adenomas in the intermediate lobe of the pituitary and healthy pituitaries were included.

Pax7-expression was mainly seen in the IL of the pituitary, but probably also, to a lesser extent, in the anterior lobe (AL). Pax7-expression appeared in clusters of positive cells, but also scattered through the tissue. Sox-expression was mainly seen scattered through the adenohipophysis. In only a minimal number of pituitary samples, Sox2-positivity in the marginal zone could be observed.

The overall hypothesis was that Pax7- and Sox2-expression is higher in adenomas and/or malignant tumors than in healthy tissue. Also, it is hypothesized that enlarged adenomas express more of these markers than non-enlarged adenomas.

For quantification of Pax7- and Sox2-expression, the percentage of positive cells was scored in each slide, to make comparison between samples possible. Somehow surprisingly, we found a higher expression of Pax7 in healthy pituitary tissue compared to microadenomas ($p=0.003$) and a higher expression of Pax7 in healthy pituitary tissue compared to malignant pituitary tumors ($p=0.036$). However, we also found that the expression of Pax7 was significantly higher in macroadenomas than in microadenomas ($p=0.007$), which is consistent with our hypothesis. No significant differences were found for Sox2 expression when comparing the different groups. More research will be needed to evaluate the correlation of Pax7- and Sox2-expression to clinical parameters such as survival and disease free interval.

Introduction

Cushing's disease

The pituitary gland is a key regulator of the endocrine balance in the adult dog. The anterior lobe (AL) of the pituitary contains five different cell types: corticotrophs, somatotrophs, lactotrophs, thyrotrophs and gonadotrophs. These cell types produce six different hormones: adrenocorticotrophic hormone (ACTH); growth hormone (GH); prolactin (PRL); thyroid stimulating hormone (TSH); follicle stimulating hormone (FSH) and luteinizing hormone (LH), respectively. The intermediate lobe (IL) produces alpha-melanocyte stimulating hormone (α -MSH) and the posterior lobe (PL), also called the neurohypophysis, secretes neurohormones that are produced in the hypothalamus, arginine vasopressine (AVP) and oxytocine (OT) (Rijnberk, 2010; Melmed, 2011). The pituitary gland, with the different parts (IL, AL, PL) is depicted in figure 1.

Pituitary tumors can be of 7 different histological subtypes and each subtype can be associated with different clinical signs (Hosoyama, 2010). Ten to 15 percent of the pituitary adenomas in humans are ACTH-producing tumors (or corticotroph adenomas), which results in hypercortisolism (Hosoyama, 2010). Hypercortisolism, or Cushing's syndrome, is a frequently occurring endocrine disease in dogs. In 80 to 85% of the cases, it is caused by a pituitary tumor (in that case it's called Cushing's disease) and in the other 15 to 20% of the cases, Cushing's syndrome is caused by a tumor in the adrenal gland (Kooistra, 2012). Pituitary adenomas can also have non-endocrine manifestations, these tumors are non-functioning adenomas. A corticotroph tumor with a non-endocrine manifestation is also called a silent corticotroph adenoma (SCA). These adenomas show positive immunoreactivity for ACTH but without signs of Cushing's disease. The ACTH plasma level is also normal. (Hosoyama, 2010). Pituitary tumors are usually benign and can range from microadenomas to macroadenomas and large tumors (Rijnberk, 2010).

Although the AL normally is the producer of ACTH in the pituitary, Cushing's disease not always originates from a tumor in this area. In about one-fourth to one-fifth of the cases, Cushing's disease is caused by a tumor in the IL. Tumors may also occur in both lobes (Rijnberk, 2010). This is due to the fact that both the AL and the IL contain cells that synthesize a common factor, namely prohormone POMC (proopiomelanocortin), which is first converted to active ACTH, after which it can be converted to the hormone α -MSH. In short, POMC is a protein precursor for both ACTH and α -MSH (Rijnberk, 2010).

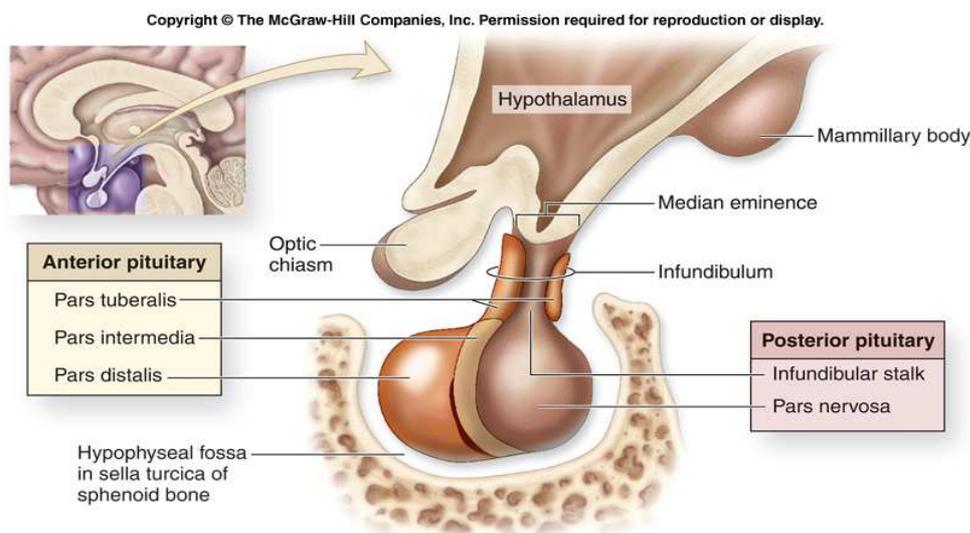


Figure 1: the pituitary gland

Diagnosis of Cushing's disease

Dogs with Cushing's disease have excessive levels of the hormones ACTH and cortisol in their blood. Production of cortisol takes place in the cortex of the adrenal gland and is regulated by ACTH. Normally ACTH is produced in the anterior lobe of the pituitary gland. In the case of a corticotroph (or melanotroph) adenoma in the pituitary gland, too much ACTH is produced, resulting in increased plasma cortisol levels. This may lead to clinical signs such as polyuria, polydipsia, polyphagia, alopecia, abdominal obesity, muscle atrophy, lethargy and diabetes mellitus (Rijnberk, 2010). It can be difficult to discriminate between pituitary- and adrenocortical-dependent hypercortisolism. If a pituitary tumor grows so large that it compresses the surrounding brain, neurological signs may develop. However, usually diagnosis of Cushing's disease is initiated by the development of endocrine clinical signs. In the Netherlands, a high dose dexamethasone suppression test is used most commonly for diagnosis, which can be performed by the owner at home. Urine samples are collected by the owner and urinary cortisol/creatinine ratios (UCCRs) are determined in the lab. If UCCRs in the first two samples are elevated, hypercortisolism is diagnosed. After collection of the second urine sample, the owner has to administer three oral doses of dexamethasone (0.1 mg per kg body weight) at 8-hour intervals (Kooistra, 2012). If suppression of UCCR in the third urine sample is greater than 50%, the diagnosis of Cushing's disease is made. If suppression is less than 50%, both pituitary-dependent and adrenocortical-dependent hypercortisolism are possible. Cushing's disease is still possible due to either a resistant tumor in the AL or a tumor in the IL, which is resistant to suppression by dexamethasone because of specific hypothalamic control of hormone synthesis in the IL (Kooistra, 2012). In the case of suppression below 50%, measurements of endogenous ACTH are necessary. In the case of a pituitary corticotroph (or melanotroph) tumor a much higher ACTH level is measured than in the case of an adrenocortical tumor, which leads to low levels of ACTH due to enhanced negative feedback. In addition, visualization by a CT-scan or abdominal ultrasound of the adrenal glands and pituitary gland helps to confirm the diagnosis. In Cushing's disease, bilateral symmetrical enlargement of the adrenal gland is seen, in the case of adrenal-dependent hypercortisolism unilateral enlargement is observed, indicative of an adrenal tumor (Rijnberk, 2010). Eventually, when the diagnosis Cushing's disease has been made, the pituitary should be visualized by means of a CT- or MRI-scan, to gain more insight into the prognosis and/or in the appearance of the tumor (pituitary size and location in relation to other structures) and to decide if surgery is the treatment of choice (Hanson, 2005; Rijnberk, 2010).

Treatment of Cushing's disease

Common medical treatments for Cushing's disease in dogs are chemotherapy with the adrenocorticolytic drug o,p'-DDD (Mitotane) or therapy with a competitive inhibitor of adrenal 3-hydroxysteroid dehydrogenase (Trilostane) (Rijnberk, 2010). Next to drug treatment, surgery has been proven to be a promising and even more effective treatment for Cushing's disease. Through transsphenoidal hypophysectomy, the whole pituitary gland is removed and in this manner the primary cause of Cushing's disease is eliminated. In the study of Hanson et al (2005), it was demonstrated that surgery eventually led to a better overall success rate than medical treatment with Mitotane. After surgery, recurrence rates were lower and the 1-, 2- and 3-year survival fractions were higher.

However, even though remission rates are relatively high after surgery, postoperative deaths still occur and recurrences are common in dogs. In the study of Hanson et al (2005), recurrence occurred in 20% of the dogs that had undergone surgery. It has been demonstrated that pituitary size is associated with postoperative survival and recurrence; dogs with larger tumors have a reduced chance of postoperative survival and an increased risk for recurrence (Hanson, 2007). Pituitaries with

adenomas can be classified as 'enlarged' or as 'non-enlarged', depending on the ratio between the height of the pituitary and the area of the brain (P/B ratio). Pituitaries with a P/B ratio lower than 0.31 are considered non-enlarged; pituitaries with a P/B ratio higher than 0.31 are considered enlarged. In 10 to 30% of the dogs with Cushing's disease the pituitary is enlarged (van Rijn, 2010).

In search for new and better treatment possibilities, pituitary stem cells may be an important target in the future. According to the cancer-stem cell theory, stem cells play an important role in pituitary tumorigenesis (Vankelecom, 2010).

The role of pituitary stem cells in pituitary tumorigenesis

The IL and the AL both are derived from the embryonal structure 'Rathke's pouch'. During embryogenesis, like in other tissues, pluripotent progenitor/stem cells in Rathke's pouch develop into a full set of terminally differentiated hormone-producing cells, which is already present at birth. Although the different hormone lineages make different cell fate choices during differentiation, like mentioned before, corticotrophs and melanotrophs both depend on the proopiomelanocortin (POMC) gene during differentiation. (Hosoyama, 2010; Budry, 2012, Rijnberk, 2010).

The cellular composition of the pituitary still undergoes many changes during early post-natal maturation and also later in life, during periods such as lactation, growth, stress and estrus. During pregnancy for example, estrogens cause an increase in lactotrophs in the pituitary, which produce PL (Nolan and Levy, 2009). This dynamic adaptation and basal cell turnover during life is thought to be driven by the recruitment and differentiation of stem/progenitor cells that are still present in the pituitary. Nolan and Levy (2006) for example, discovered that the increase of corticotrophs in the pituitary after adrenalectomy is for the largest part due to differentiation of a pool of pre-existing hormonally null cells and only for a minor part due to replication of existing ACTH cells.

For tumors in general, compelling evidence is present that stem cells and progenitor cells play an important role in tumorigenesis. This gave rise to the theory that tumor growth is mainly dependent on a small population of cells in the tumor with stem cell-like properties, so-called cancer stem cells (CSCs). According to this theory it is also proposed that treatment of malignant tumors is only successful if it results in complete eradication of these CSCs. The origin of these CSCs is still unknown. They can arise from transformed normal stem cells, progenitor cells or even differentiated normal or tumor cells that got back stem cell properties. For this reason, identification and characterization of tumor cells with stem cell-like behavior has become a major issue (Vankelecom, 2010).

Much research has been done on possible pituitary progenitor/stem cells in mice and humans. For a cell population to be recognized as a 'pituitary stem cell population' it has to possess several features: the cells have to possess a pluripotent capacity, eternal self-renewal capacity and lack of specialization. Several pituitary stem cell candidates have been proposed, including the Folliculostellate cells (FSC), the Marginal Zone cells (MZ), lining the cleft/lumen between IL and AL, the Side Population cells (SP) discovered through fluorescent activated cell sorting (FACS), Sox2-positive/Sox9-negative cells and GFRa2/Prop1-positive stem (GPS) cells (Nassiri, 2013). However, none of these candidates has been accepted as definite stem cell population yet, because they do not meet all the requirements that are necessary for being acknowledged as stem cells. Furthermore, various studies seem to confirm that Nestin-expressing cells correspond to adult stem cells of the anterior pituitary (Gleiberman, 2008; Hosoyama, 2010). However, according to the results of the study of Gleiberman (2008), these adult Nestin-expressing stem cells do not play a significant role in the embryonic development of the anterior pituitary, but start functioning and contributing prominently to the gland soon after birth.

In search for cancer stem cells that are involved in pituitary adenomas in humans and mice, several markers have already been identified, which play an important role in pituitary development, among which Pax7 and Sox2 (Hosoyama, 2010; Budry, 2012; Menks, 2011; Alatzoglou, 2009; Kelberman, 2008). Both Pax7 and Sox2 are transcription factors that are expressed in pituitary progenitor cells. By analyzing the expression of such markers, our understanding about the role of progenitor-/stem-cells in tumorigenesis can be improved and that can be a taking point for the development of new treatment possibilities.

The role of Pax7 in pituitary tumors

In general, Pax transcription factors are important for embryonic patterning and postnatal stem cell renewal of many organs, such as the eye and the muscle. In the muscle, Pax7 is specifically expressed in satellite cells; Pax7 is involved in maintaining this tissue's specific stem cell population (Hill, 1991; Seale, 2000). Concerning the pituitary gland, the cells that express transcription factor Pax7 are mainly restricted to the IL but are also, to a small extent, found in the lumen (cleft) between AL and IL (Hosoyama, 2010). Pax7 is located in the nuclei of the cells.

According to a theory of Hosoyama et al (2010), pituitary stem cells (which are thought to be Nestin+) can differentiate to Pax7+ progenitors of ACTH expressing cells. These early progenitor cells can migrate through the IL to eventually differentiate to melanotrophs. Also, another proportion of Pax7+ cells, nearby the lumen, can migrate to the AL to differentiate to other endocrine cells, including corticotrophs. Pax7 is found to be a positive regulator of melanotroph commitment of cells that are differentiating. It activates melanotroph-specific genes and represses corticotroph-specific genes. Inactivation of the Pax7 gene results in loss of melanotroph gene expression and ceases the repression of corticotroph genes (Budry, 2012).

In humans, Pax7 expression was found in different types of ACTH adenomas; functioning corticotroph tumors, but also non-functioning corticotroph tumors. It has already been suggested that the early Pax7-positive progenitor cell population in the intermediate lobe of the pituitary gives rise to silent corticotroph adenomas in humans (Hosoyama, 2010). In dogs it has been reported that approximately 30% of pituitary corticotroph adenomas express Pax7 (Budry, 2012). Therefore it is interesting to examine the clinical significance of Pax7 in canine pituitary adenomas. Research on expression of this marker in pituitary adenomas can elucidate the role and the significance of Pax7 in pituitary tumorigenesis and the utility of this marker in therapeutical interventions for pituitary adenomas (that possibly rely on melanotroph-specific functions, concerning the fact that Pax7 stimulates differentiation towards the melanotroph lineage).

The role of Sox 2 in pituitary tumors

The large family of proteins to which Sox2 belongs is very similar to the Sry (sex-determining region of chromosome Y) protein, and has a conserved high mobility group (HMG) DNA binding domain. There are 20 Sox proteins that are widely expressed in both embryonic and adult tissues of mammals. SOX transcription factors are expressed in many cell types at early stages of developmental processes that range from maintenance of pluripotency and determination of cell fate to neurogenesis, sex development, chondrogenesis and hematopoiesis (Alatzoglou, 2009).

Sox2 is a member of the SOXB1 group; SOXB1 proteins are widely expressed in the developing neural system. SOXB1 proteins are expressed in pluripotent or undifferentiated cells and their expression is downregulated when the cells are committed to their cell fate (Alatzoglou, 2009).

Sox2 is a crucial transcription factor necessary for the function of multiple stem cell populations, especially in the developing CNS. During embryogenesis, Sox2 interacts with a complex network that consists of several other transcription factors and signaling molecules (Alatzoglou, 2009; Nassiri, 2013). Together with other transcription factors it maintains the pluripotent capacity of embryonic stem cells (Gretchen et al, 2009). An example of a transcription factor that Sox2 interacts with is Oct4, also a crucial factor during embryonic development. In embryonic stem cells, Sox2 heterodimerizes with Oct4, and together they bind to a DNA sequence present in the promoter region of their transcriptional target genes. One of these genes is pluripotential cell-specific gene *Nanog*, which encodes a transcription factor necessary for maintaining the undifferentiated state of stem cells (Kuroda et al, 2005). An example of a signaling pathway that is regulated by the activity of Sox2 is the Wnt- β -catenin pathway, a pathway that plays an important role in the development of pituitary tissue. Sox2 has been found to antagonize the transcriptional activity β -catenin (Kelberman, 2008).

Concerning pituitary development, during embryogenesis, Sox2 expression becomes restricted to Rathke's pouch. In the adult gland, Sox2 is maintained in a small population of cells lining the pituitary cleft or scattered through the AL parenchyma. These cells maintain their potential to differentiate into all pituitary cell types, representing progenitor cells (Alatzoglou, 2009; Nassiri, 2013). Because Sox2 is a transcription factor, it is mainly located in the nuclei of cells, but it can also be found in the cytoplasm of cells (Gretchen et al, 2009).

As mentioned before, Sox2⁺/Sox9⁻ cells are being recognized as a potential stem cell population, because they do not produce hormones, divide slowly, express Stem cell antigen 1 (Sca1), can differentiate and can form secondary pituispheres, which are all stem cell-like properties. However, self-renewal capacity has yet to be further investigated for this cell population (Nassiri, 2013).

The marker Sox2 has already been linked to tumors in the lungs, breast and brain and could also play a role in pituitary tumorigenesis (Dong, 2004; Chen, 2008; Phi, 2008). Since Sox2 is such a crucial factor in pituitary development and seems to be associated with a certain progenitor/stem cell population, it would be interesting to examine the clinical significance of this marker in pituitary adenomas. Analyzing the expression of this marker could make its possible role in pituitary tumorigenesis more clear and could say something about the utility of this marker in the development of therapeutical interventions for pituitary adenomas.

Pax7⁺/Sox2⁺ cell population(s)

It has been proposed by Budry et al (2012) that during differentiation, Sox2 expression precedes Pax7 expression in progenitor cells in the IL. Differentiation of the Pax7-positive cells in the IL, driven by transcription factor Tpit, leads to cells with a melanotroph-specific program.

According to the same study, early after birth (postnatal day 2) a certain overlap exists in Sox2⁻ and Pax7⁻ expression in the IL. As mentioned before, Sox2-positive cells are found in the marginal zone, lining the cleft. The remainder of the cells in the IL are occupied by Pax7-positive cells. Some scattered cells in this area however, were found to express both markers. When transcription factor Tpit is not present (Tpit^{-/-}), this overlap is more widespread. Most cells without Tpit (Tpit^{-/-}) are found to be positive for both Sox2 and Pax7. These cells cannot fully exit their progenitor state and maintain Sox2 in the presence of Pax7.

The aim of this study

In the current study, the expression of the markers Pax7 and Sox2 in canine pituitary adenomas is investigated, by means of immunohistochemistry. A general description of the appearance of Pax7- and Sox2- expression will be given and it will be examined if expression of Pax7 and Sox2 is different (higher/lower) in different kinds of pituitary (tumor) tissue; macroadenomas, microadenomas, malignant tumors, adenomas in the IL and healthy pituitaries.

The overall hypothesis is that Pax7 expression is higher in (specifically IL-)adenomas and/or malignant tumors than in healthy tissue and that Sox2 expression is higher in adenomas and/or malignant tumors than in healthy tissue. Also, it is hypothesized that enlarged adenomas express more of these markers than non-enlarged adenomas.

This research may provide more knowledge about and insight into pituitary progenitor/stem cells and their role in tumorigenesis. This may eventually lead to better understanding of pituitary tumorigenesis, which can be used for the development of new treatment methods.

Materials & methods

Animals

In this research project, samples of 50 pituitary tumors and 10 normal pituitary glands from a total of 60 dogs were used (Table 1).

The concerning pituitary tissues have been collected in dogs with confirmed Cushing's disease undergoing transsphenoidal hypophysectomy, as their first line of treatment. Surgery was performed in the Small Animal University Clinic in Utrecht. Cushing's disease was diagnosed by means of a high dose dexamethason suppression test, if necessary followed by measurements of endogenous ACTH, as explained before.

Normal pituitary tissues were collected from healthy dogs that were terminated in other unrelated experiments for which DEC was obtained.

Clinical, biochemical, imaging, hormonal and follow-up data of the dogs with Cushing's disease were available from the medical records (Vetware) and from the pituitary database of Dr. B. Meij and are presented in Table 1 and 2.

Patient selection was based on clinical parameters and results of histopathology. There were non-enlarged pituitaries, enlarged pituitaries, adenomas from the intermediate lobe and tumors with malignant characteristics. 18 tumor samples localized in the AL with a P/B ratio varying from 0.20 – 0.30 were collected; 20 tumor samples localized in the AL with a P/B ratio varying from 0.63 – 1.40; 7 tumor samples localized in the IL; and 5 tumor samples localized in the AL that showed malignant characteristics, such as infiltrating tumor tissue and the presence of mitoses (Table 1).

Histology and immunohistochemistry

Specimens of pituitary tissue removed during surgery were fixed in 4% methanol-formaldehyde, embedded in paraffin, and consecutive sections were used for histology (hematoxylin and eosin [H&E] staining), hormone immunohistochemistry, and staining for Pax7 and Sox2. The diagnosis of pituitary neoplasia was recognized on H&E staining by its typical adenomatous organization and characteristic basophilic-eosinophilic-chromophobic staining pattern in comparison with normal pituitary tissue. The diagnosis of corticotroph adenoma was confirmed by immunostaining for ACTH, melanocyte-stimulating hormone (MSH), and growth hormone (GH) as previously described (Van Rijn et al 2010).

Expression of Pax7 and Sox2 in pituitary corticotroph adenomas in dogs

Table 1 – Clinical characteristics of patients

No.	Group	Age	Sex	Weight	Breed	C/C	% suppression	Pituitary size (mm) (H x W x L)	P/B
1	NE	5.2	M	38	american staffordshire terrier	69.0	93.3	5.6 x 7.2 x 6	0.29
2	NE	9.4	M	8.35	cross-breed 1	32	96.9	3.2 x 4 x 4	0.23
3	NE	7.9	M	8	bolognese	64.5	96.9	3 x 4.2 x 4	0.20
4	NE	9.6	F	8.9	dachshund	13.3	65.4	4 x 5 x 7	0.29
5	NE	8.3	F	5.9	maltese	15.5	87.1	4 x 5 x 5	0.25
6	NE	9.2	F	15	cross-breed 1	59.5	97.8	5 x 6.7 x 7	0.28
7	NE	10.0	F	38.18	siberian husky	30.6	78.4	6 x 9 x 10	0.30
8	NE	6.1	F	50	bordeaux dog	36	91.4	6 x 9 x 10	0.26
9	NE	6.7	M	8,2	yorkshire terrier	42	96.7	4 x 4 x 4	0.24
10	NE	8.8	M	24.7	Irish terrier	29.5	93.6	5 x 7 x 6	0.30
11	NE	9.2	F	36.0	labrador retriever	127.7	85.0	4.9 x 5.2 x 6.2	0.29
12	NE	2.7	F	10.7	king charles spaniel	100	96.0	2 x 6.2 x 4.5	0.29
13	NE	10.2	M	33.4	boxer	14.5	84.8	5.8 x 4 x 7	0.27
14	NE	9.6	M	4.8	maltese	100.7	97.5	3.4 x 3.8 x 3.2	0.24
15	NE	8.9	M	7.4	australian terrier	13.85	79.1	4.6 x 5.8 x 5.7	0.3
16	NE	5.6	M	21.0	beagle	19.6	93.9	4.2 x 5 x 6	0.26
17	NE	5.9	M	15.8	king charles spaniel	383.15	95.1	6.1 x 6.8 x 6.6	0.29
18	NE	8.4	F	6.4	maltese	57.35	95.8	3.6 x 3.8 x 2.2	0.25
19	E	3.9	M	22.8	english cocker spaniel	64.5	-58.1	15 x 17 x 18	0.76
20	E	8.6	M	33.4	boxer	44.9	92.4	12.4 x 11.6 x 11	0.70
21	E	6.1	M	48.2	golden retriever	13.2	87.1	18.3 x 19.6 x 17.2	0.95
22	E	11.7	M	13.0	jack russell terrier	13.5	74.1	12.6 x 13.4 x 16.2	0.74
23	E	12.1	M	24.0	beagle	NA	NA	22.5 x 21.3 x 20.7	1.25
24	E	7.3	M	23.0	cross-breed 2	0.0	NA	10.4 x 11 x 11.3	0.65
25	E	8.2	M	23.2	beagle	74.5	75.8	16.6 x 14.6 x 16	1
26	E	6.5	M	12.8	cross-breed	81.0	60.5	14.9 x 13.8 x 16.1	0.99
27	E	7.1	M	41.5	chesapeake bay retriever	65.0	66.2	11.9 x 13.9 x 16.5	0.7
28	E	8.2	F	31.7	belgian shepherd malinois	32.5	-50.8	18.4 x 17.5 x 14.9	1.02
29	E	10.0	M	21.5	english staffordshire terrier	18.1	59.6	11.5 x 12.2 x 10.5	0.64
30	E	7.0	M	10.4	french bulldog	417.3	NA	11.5 x 11.1 x 9.5	0.63
31	E	10.7	M	18.5	border collie	55	61.8	11.1 x 9.8 x 10.1	0.66
32	E	7.1	F	44.9	american bulldog	45.5	31.4	13.7 x 15.8 x 14.8	1.38
33	E	9.2	F	37.6	rhodesian ridgeback	14.8	47.3	13.7 x 13.1 x 13.4	0.78
34	E	9.7	M	24.8	cross-breed	NA	NA	18.9 x 25.6 x 21.6	1.15
35	E	7.6	F	22.3	labrador retriever	NA	NA	14.7 x 9.6 x 9.1	0.93
36	E	9.9	F	29.1	labrador retriever	104.5	4.3	11.4 x 14.5 x 13.2	0.71
37	E	8.1	F	12.6	cross-breed	20.5	73.7	19.3 x 15.3 x 15.5	1.4
38	E	11.0	M	10.0	shih tzu	NA	NA	10.6 x 15 x 12.9	0.67

Expression of Pax7 and Sox2 in pituitary corticotroph adenomas in dogs

39	MA	10.1	M	25.2	cross-breed 2	57.0	94.4	11.5 x 10.6 x 10	0.70
40	MA	5.8	F	16.7	english cocker spaniel	96.1	69.4	14.2 x 17.4 x 17	1.10
41	MA	11.9	F	23.0	german wirehaired pointer	81.5	14.1	7.6 x 8.7 x 11.4	0.47
42	MA	8.4	F	33.2	boxer	126.55	39.8	10.4 x 12 x 8.1	0.49
43	MA	9.3	F	40.0	boxer	16.5	52.7	11.7 x 12.4 x 13.9	0.7
44	ILA	9.4	F	19.5	german shorthaired pointer	59.0	92.4	7.4 x 8.6 x 8	0.45
45	ILA	8.3	F	36,5	labrador retriever	30.7	88.3	11.5 x 11.8 x 11.6	0.56
46	ILA	9.1	F	18,3	cross-breed	53.5	51.4	6.4 x 6.7 x 7.6	0.41
47	ILA	6.9	M	32,9	cross-breed	133.9	89.7	7.5 x 9.1 x 8	0.45
48	ILA	7.4	M	31,0	golden retriever	108.5	84.3	16.5 x 19.7 x 12.4	0.76
49	ILA	10.4	F	19,7	beagle	361.45	47.0	7.7 x 7.4 x 9.8	0.48
50	ILA	10.7	M	25,5	cross-breed	126	31.0	17.4 x 17.6 x 21.2	0.74
51	H	8m	M	31	Bouvier des Flandres	NA	NA	NA	NA
52	H	3j8m	F	N/A	Labrador Retriever	NA	NA	NA	NA
53	H	3j	M	N/A	Labrador Retriever	NA	NA	NA	NA
54	H	2j9m	M	N/A	Labrador Retriever	NA	NA	NA	NA
55	H	3j1m	F	N/A	Labrador Retriever	NA	NA	NA	NA
56	H	2j9m	F	N/A	Labrador Retriever	NA	NA	NA	NA
57	H	2j9m	M	N/A	Labrador Retriever	NA	NA	NA	NA
58	H	2j	M	11.7	Beagle	NA	NA	NA	NA
59	H	2j	M	14.3	Beagle	NA	NA	NA	NA
60	H	NA	NA	N/A	NA	NA	NA	NA	NA

P/B ratio: pituitary-height/brain-area ratio; NE: non-enlarged adenomas (< 0.31); E: enlarged adenomas (> 0.31); MA: malignant tumor; ILA: intermediate lobe adenoma; H: healthy dog; NA: not available/not applicable; C/C: average of UCCRs of day 1 and 2; % suppression: % suppression by dexamethasone; F: female; M: male.

Optimization of immunohistochemistry protocols

Because hardly any other immunohistochemistry (IHC) experiments had been done on the expression of Pax7 and Sox2 in pituitary tissue in dogs yet, first optimization of the IHC protocols for Sox2 and Pax7 was necessary, among others to determine which antibody concentrations were optimal to use for these markers in pituitary tissue of dogs.

Slides from two different paraffin blocks were used for optimization. One block was available for Pax7 (which included tissue from a tumor in the IL) and one block was available for Sox2 (which included tissue from a healthy dog). Sections (4 µm) were mounted on poly-L-lysine-coated slides.

The Sox2 and Pax7 antibodies that were used were both from Cell Signaling Technology, Inc.

For optimization of the Pax7 protocol, recommendations concerning antibody concentration and IHC protocol from other studies that examined the expression of Pax7, were taken into account (Budry, 2012; Canadian colleague Jacques Drouin). For Sox2, recommendations from the company where the Sox2 antibody was bought (© 2012 Cell Signaling Technology, Inc.) were taken into account during optimization of the protocol.

At the end of optimization, for Pax7 the optimal antibody concentration turned out to be 1:20; for Sox2, the chosen antibody concentration was 1:800.

IHC for Pax7 and Sox2

Immunohistochemistry protocols for Sox2 and Pax7 can be found in Appendix 1.

For immunohistochemistry, 4 µm thick sections were mounted on poly-L-lysine-coated slides and deparaffinized. Antigen retrieval was performed in a citrate bin (10 mM Citrate with 0.05% Tween 20, pH 6), placed in a water bath (98°C). The citrate bin with the slides used for Pax7 immunostaining were placed in the water bath for one hour; the citrate bin with the slides used for Sox2 immunostaining were placed in the water bath for 30 minutes. After this, slides were left to cool down to room temperature for 20 min. Slides were washed 2 x 5 minutes in PBS. Blocking was performed with peroxidase, endogenous enzyme block and, after washing in PBS, slides were treated for 30 minutes with 10% normal goat serum, diluted in antibody diluent (1:10). Incubation with the primary antibody took place over night at 4°C, for both Pax7 and Sox2. The anti-Pax7 monoclonal mouse antibody was diluted 1:20 in antibody diluent and the anti-Sox2 monoclonal rabbit antibody was diluted in 1:800 antibody diluent (© 2012 Cell Signaling Technology, Inc.).

The next day, after the slides were washed 3 x 5 minutes in PBS on a mechanical shaker, they were incubated with the secondary antibodies for 30 minutes: labeled polymer-HRP anti-mouse for Pax7 immunostaining and labeled polymer-HRP anti-rabbit for Sox2 immunostaining (Dako Envision Systems). Slides were washed 3 x 5 minutes in PBS on a mechanical shaker, before the detection of staining with DAB-substrate (Vector laboratories). After being washed for 5 minutes in PBS, the slides were counterstained for 10 seconds with hematoxylin and eosin (HE) and washed for 10 minutes in running tap water. Slides were dehydrated with a series of graded alcohol and xylene and mounted with Vectamount (Vector Laboratories).

Positive immunostaining controls were obtained by using slides with the tissues used for the optimization of the protocols for Pax7 and Sox2. Negative controls were obtained by omitting the primary antibody.

Analysis of Pax7- and Sox2-expression

Expression patterns were evaluated in the different slides and descriptions of typical patterns were made. Also, the expression of the markers Pax7 and Sox2 in each slide was quantified by categorizing them by estimated percentages. 6 categories were used (Table 2).

Table 2. Percentage-categories of marker-expression

0	0%
1	< 5%
2	5 - 10%
3	11 - 30%
4	31 - 60%
5	> 60%

The expression of each slide was classified into one of these categories. The percentage represents the percentage of positive cells of the whole adenohiphophysis-tissue (AL + IL) present in the slide (so neurohiphophysis tissue was left out).

Scoring of the slides was done randomly and by two different persons (J. van der Bend and Drs. S. van Rijn), to make the results more reliable. The persons who scored the slides did not know what kind of tissue was present in which slide.

Statistical analysis

For the comparison of the expression of Pax7 and Sox2 between different groups, the Mann-Whitney U test was used. This test was chosen because the variables are ordinal and because in each test, two independent groups are compared. Significance was set at $p < 0.05$.

Results

Description of Pax7- and Sox2- expression

Pax7-expression

Pax7 expression was seen in the nuclei of cells. Pax7 was expressed in the adenohipophysis only. It was, however, not always clear if only the intermediate lobe of the adenohipophysis contained positive cells. Sometimes, in the AL there appeared to be cells present expressing Pax7 as well, in both tumor tissues and healthy tissues.

Pax7-expression was seen in different appearances. In some slides, positive cells scattered through the tissue (sometimes only a minimal number of those), were seen. In other slides, (a) large cluster(s) of positive cells were found in a higher density. Furthermore, often the Pax7-positive cells that appeared in groups seemed to be colored darker brown than the scattered Pax7-positive cells.

In some slides, it was clear that the cluster(s) with Pax7-positive cells was/were located in the IL, because it was tissue that was situated next to the neurohypophysis on one side, and sometimes even with the lumen/cleft on the other side (Figure 2). In many slides however, it was not really clear in what kind of tissue the Pax7-positive cells were located, because the structure of the tissue was not really intact anymore (Figure 3).

When comparing the presence of high density- cluster(s) of Pax7-positive cells between the different groups, it was noted that the tumor samples in the IL contained the most slides in which such clusters were present. 71% of those slides (5 out of 7) contained these clusters, while for example in only 40% (4 out of 10) of the slides with healthy tissue these clusters were present (Table 3).

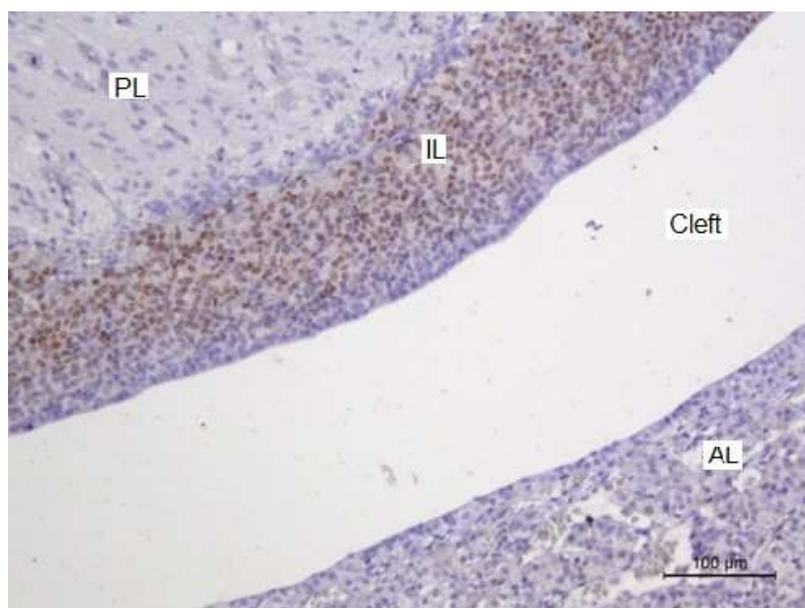


Figure 2: Pax7-expression in healthy pituitary tissue. Pax7-positive cells can be observed in the IL (intermediate lobe). The other tissues; the AL (anterior lobe) and the PL (posterior lobe) are Pax7-negative.

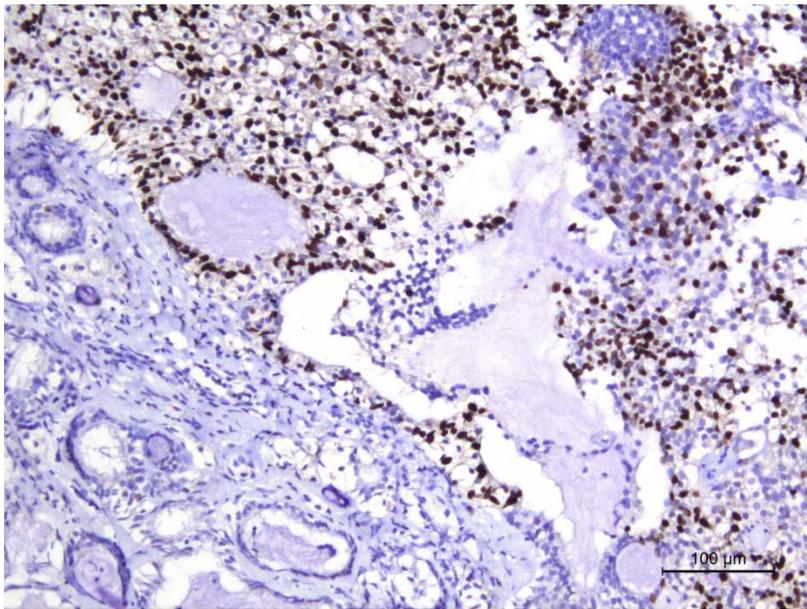


Figure 3: Pax7-expression in IL-adenoma tissue

Two different tissues are observed, with the adenoma-tissue in upper/right part, which mainly represents intermediate lobe tissue. The brown cells are positive for Pax7. Various cells in this IL-/adenoma-tissue are positive for Pax7, but there are also cells negative for Pax7.

Sox2-expression

Sox2 expression was seen in the adenohipophysis, mostly in the anterior lobe-tissue (Figure 4). Also, in an exceptional case, a row of Sox2-positive cells could be observed in the marginal zone around the cleft. This was however not seen in many slides, probably because often the structure of the tissue was not intact and recognizable anymore and/or not all parts of the pituitary were present. Furthermore, in several slides, also cells in the neurohypophysis were found to be positive for Sox2 (Figure 4). These appeared to be stroma cells.

The Sox2-positive cells in the adenohipophysis were mostly scattered through the whole tissue. Despite the sometimes high number of scattered positive cells, the coloring did seem to be specific. Besides the scattered Sox2-positive cells, often also many Sox2-positive cells were found lining the (small) gland-ducts in the adenohipophysis (Figure 4). Also stroma cells, often recognized by their flattened cell nuclei, were sometimes positive for Sox2 (Figure 4).

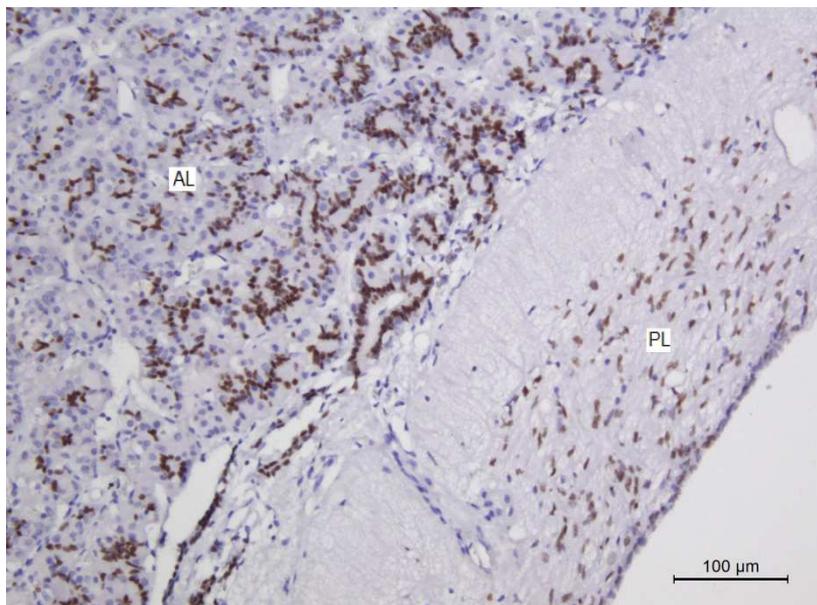


Figure 4: Sox2-expression in healthy pituitary tissue

Sox2-positive cells are located in the anterior lobe (AL) (multiple Sox2-positive cells are lining small gland-ducts) and also in the posterior lobe (PL).

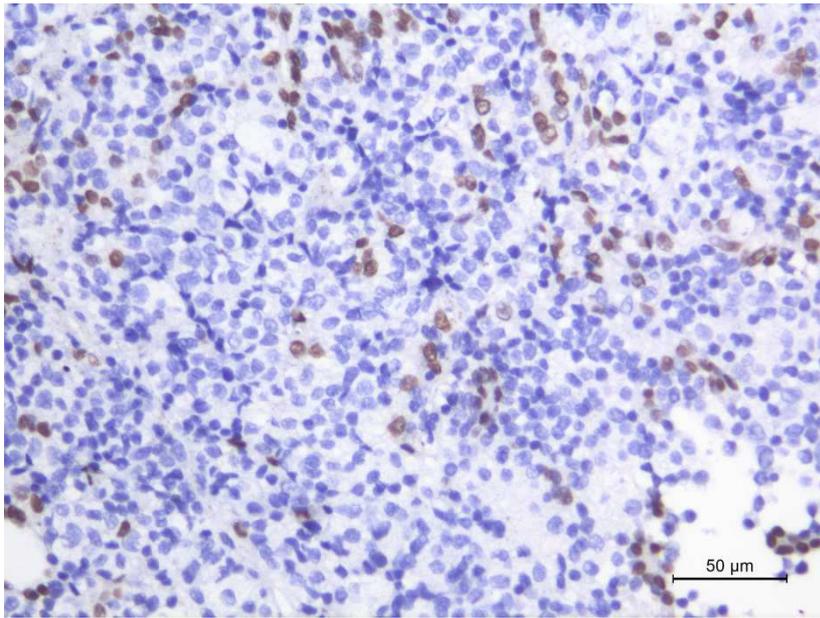


Figure 5: Sox2-expression in an enlarged adenoma
Sox2-positive cells are located in the adenoma-tissue in the adenohipophysis

Quantification of Pax7- and Sox2- expression

Each slide was classified into one of the six categories mentioned before, by two different persons (J. van der Bend and Drs. S. van Rijn). From the total of 120 slides, of both Sox2 and Pax7, 71 slides were classified in the same category by the two persons who scored the slides. The remaining 49 slides were scored differently (25 of the Pax7-slides and 24 of the Sox2-slides), and for these slides, the scores of only one person (J. van der Bend) were used.

Because, as mentioned before, in several slides different Pax7-positive cell populations seemed to be present, namely, clusters of (often dark brown) cells and also (often lighter brown) cells scattered through the tissue), it was noted with ‘C’ and/or a ‘S’ if the slide contained clusters (C) of positive cells and/or scattered (S) positive cells. If a slide contained a cluster of positive cells, it was also noted behind the ‘C’, between brackets, in which category this specific cell population was classified (Table 3).

Table 3 – Follow up and expression-scores

No.	Group	follow up (months)	dfi (months)	Rem_cens (1=rec, 0=no rec)	Rem (1=yes, 0=no)	Sur_cens (1=dead, 0=cushing)	Dead (1=dead, 0=alive)	Pax7 Expr.	Pax7 C/S	Sox2 Expr.
1	NE	17.9	14.4	0	1	1	1	0	-	2
2	NE	23.8	23.8	0	1	0	1	1	C (4/5)	3
3	NE	50.8	50.8	0	1	0	1	2	C (4) + S	3
4	NE	0.0	NA	NA	NA	0	1	1	C (5)	2
5	NE	33.6	33.6	0	1	0	0	1	S	3
6	NE	39.0	39.0	0	1	0	1	0	-	3
7	NE	22.4	22.4	0	1	0	1	1	C (5) +S	3
8	NE	8.7	8.7	0	1	0	1	1	C (4)	3
9	NE	125.9	125.9	0	1	0	0	0	-	3
10	NE	19.8	NA	0	0	1	1	2	S	0

Expression of Pax7 and Sox2 in pituitary corticotroph adenomas in dogs

11	NE	51.6	51.6	0	1	0	1	1	S	3
12	NE	0.0	NA	NA	NA	1	1	1	S	3
13	NE	14.3	14.3	0	1	0	1	0	-	2
14	NE	18.2	18.2	0	1	0	0	1	C (4/5) +S	3
15	NE	8.7	7.7	0	1	0	1	0	-	3
16	NE	16.6	16.6	0	1	0	0	1	S	1
17	NE	16.1	16.1	0	1	0	0	0	-	3
18	NE	12.7	12.7	0	1	0	0	1	C (4) + S	4
19	E	2.6	1.5	1	1	1	1	1	S	1
20	E	23.8	23.8	0	1	0	1	1	S	3
21	E	15.3	15.3	0	1	0	1	1	S	1
22	E	20.6	8.4	1	1	1	1	1	S	2
23	E	0.0	NA	NA	NA	1	1	2	S	2
24	E	28.6	28.6	0	1	0	1	4	C (5) + S	4
25	E	61.2	40.1	1	1	0	0	1	S	3
26	E	18.8	4.4	1	0	1	1	2	C (4) + S	2
27	E	28.1	19.6	1	0	1	1	0	-	0
28	E	15.0	4.4	1	0	0	0	2	S	3
29	E	19.3	19.3	0	1	0	1	1	S	2
30	E	6.0	5.9	1	0	0	0	2	S	3
31	E	43.7	43.7	1	1	0	0	1	S	2
32	E	5.5	NA	NA	NA	0	1	1	S	2
33	E	27.9	27.9	0	1	0	1	4	S	4
34	E	0.1	0.1	NA	NA	1	1	5	C (5)	1
35	E	29.3	29.3	0	1	0	1	2	S	3
36	E	26.2	18.2	1	1	1	1	1	S	3
37	E	0.1	0.1	NA	NA	1	1	1	S	2
38	E	18.4	18.4	0	1	0	0	1	S	3
39	MA	43.9	30.0	0	1	0	1	0	-	0
40	MA	27.1	18.9	1	1	0	1	1	S	2
41	MA	27.4	27.4	0	1	0	1	1	C (4) + S	3
42	MA	14.7	14.7	1	1	1	1	1	C (4) + S	4
43	MA	5.9	5.9	1	1	1	1	1	S	2
44	ILA	66.5	49.8	1	1	1	1	3	C (5) + S	2
45	ILA	49.5	49.5	0	1	0	0	0	-	0
46	ILA	5.6	5.6	0	1	0	1	2	C (4/5) + S	3
47	ILA	64.9	64.9	0	1	0	0	1	C + S	2
48	ILA	5.8	NA	NA	0	1	1	1	C (4) + S	2
49	ILA	15.9	15.9	0	1	0	0	2	C (4) + S	4
50	ILA	0.4	NA	NA	NA	NA	NA	0	-	3
51	H	NA	NA	NA	NA	NA	NA	2	S	1
52	H	NA	NA	NA	NA	NA	NA	3	C (5)	4

Expression of Pax7 and Sox2 in pituitary corticotroph adenomas in dogs

53	H	NA	NA	NA	NA	NA	NA	0	-	0
54	H	NA	NA	NA	NA	NA	NA	4	S	4
55	H	NA	NA	NA	NA	NA	NA	1	S	3
56	H	NA	NA	NA	NA	NA	NA	2	S	3
57	H	NA	NA	NA	NA	NA	NA	1	S	3
58	H	NA	NA	NA	NA	NA	NA	4	C (5)	4
59	H	NA	NA	NA	NA	NA	NA	3	C (5) + S	4
60	H	NA	NA	NA	NA	NA	NA	2	C (5) + S	4

P/B ratio: pituitary-height/brain area ratio; NE: non-enlarged adenoma (P/B < 0.31); E: enlarged adenoma (P/B > 0.31); MA: malignant tumor; ILA: intermediate lobe adenoma; H: healthy tissue; NA: not available/not applicable; dfi: disease free interval; Rem(_cens): remission (censored); rec: recurrence; Sur_cens: survival censored; Pax7 C/S: Clustered and/or Scattered Pax7-positive cells; Pax7/Sox2 Expr.: category 0-5, see Materials & methods.

Pax7-expression

When all groups were mutually compared for Pax7-expression, for three comparisons significant differences were found (Figure 6c-e). First of all, healthy tissues express significantly more Pax7 than non-enlarged pituitaries and tumors with malignant characteristic; adenomas in enlarged pituitaries expressed significantly more Pax7 than adenomas in non-enlarged pituitaries (Table 4). No significant differences were found between Pax7-expression in IL-adenomas and healthy pituitaries (Figure 6a) and between IL-adenomas and non-IL-adenomas (Figure 6b).

Figure 6a-e: Distribution of Pax7-expression in the different categories

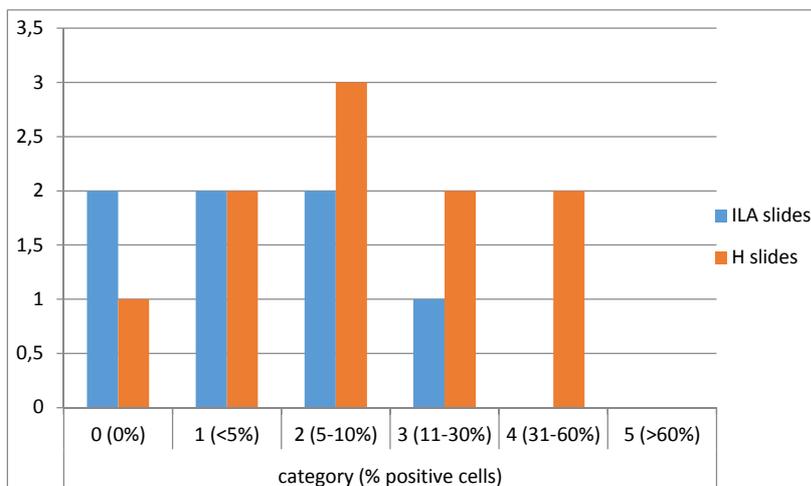


Figure 6a: Intermediate lobe adenomas (ILA) vs. healthy pituitaries (H)

Mann-Whitney U test statistic: 21
p-value: 0.161

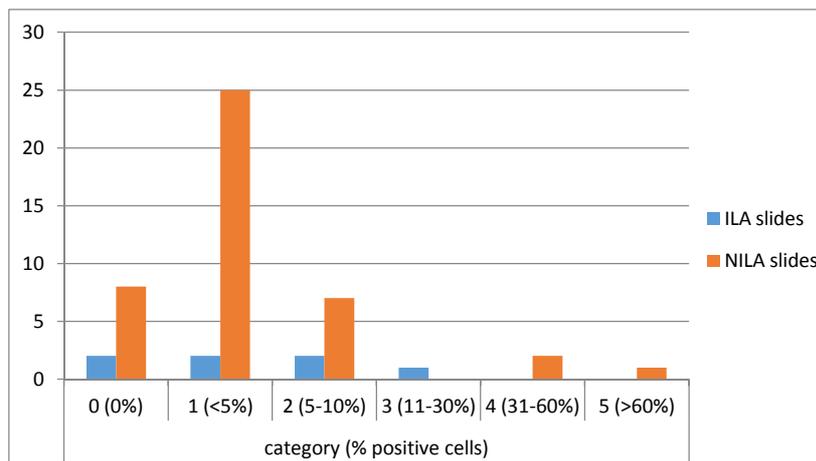


Figure 6b: Intermediate lobe adenomas (ILA) vs. other tumor types (NILA)

Mann-Whitney U test statistic: 139
p-value: 0.724

Expression of Pax7 and Sox2 in pituitary corticotroph adenomas in dogs

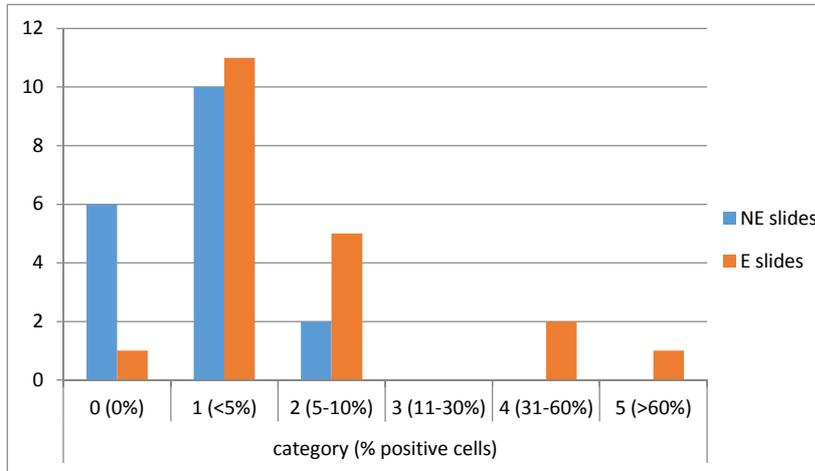


Figure 6c: Non-enlarged adenomas (NE) vs. Enlarged adenomas (E)

Mann-Whitney U test statistic: 97
p-value: 0.007

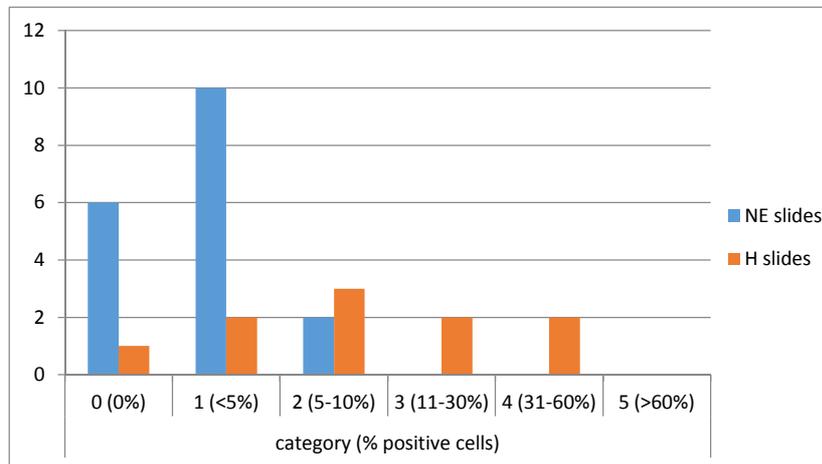


Figure 6d: Non-enlarged adenomas (NE) vs. healthy pituitaries (H)

Mann-Whitney U test statistic: 32
p-value: 0.003

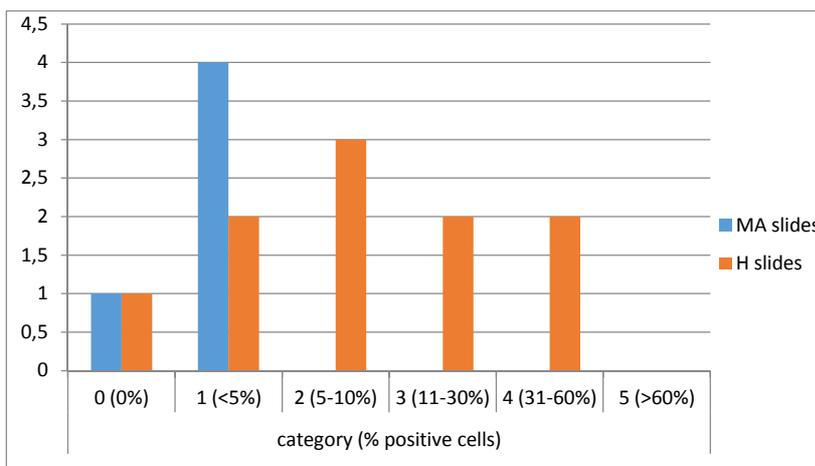


Figure 6e: Malignant tumors (MA) vs. healthy pituitaries (H)

Mann-Whitney U test statistic: 8.5
p-value: 0.036

Sox2-expression

No significant differences were found when comparing Sox2-expression in the different groups (Table 4; Figure 7a-d).

Figure 7a-d: Distribution of Sox2-expression in the different categories

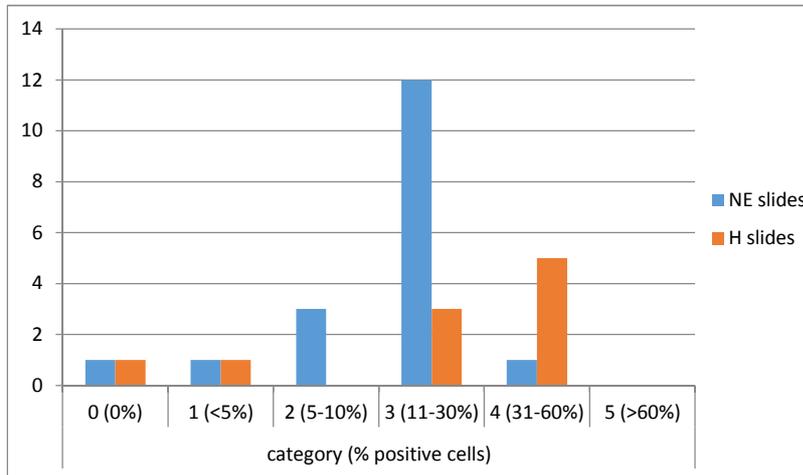


Figure 7a: Non-enlarged adenomas (NE) vs. healthy pituitaries (H)

Mann-Whitney U test statistic: 57.5
p-value: 0.088

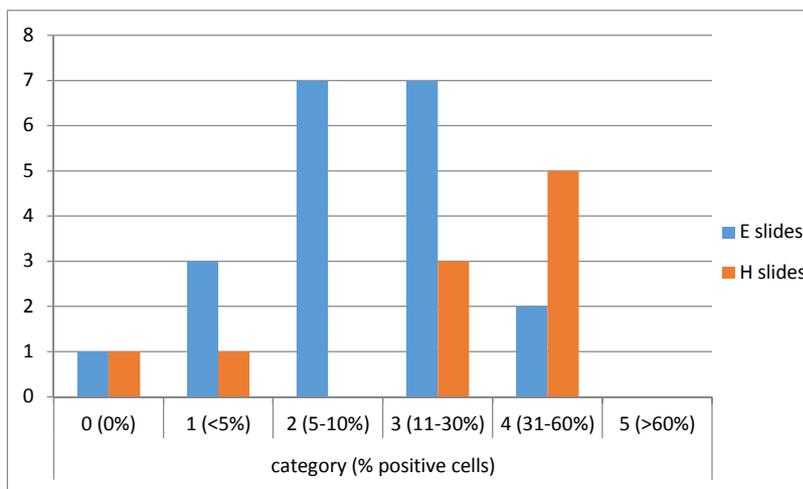


Figure 7b: Enlarged adenomas (E) vs. healthy pituitaries (H)

Mann-Whitney U test statistic: 58.5
p-value: 0.059

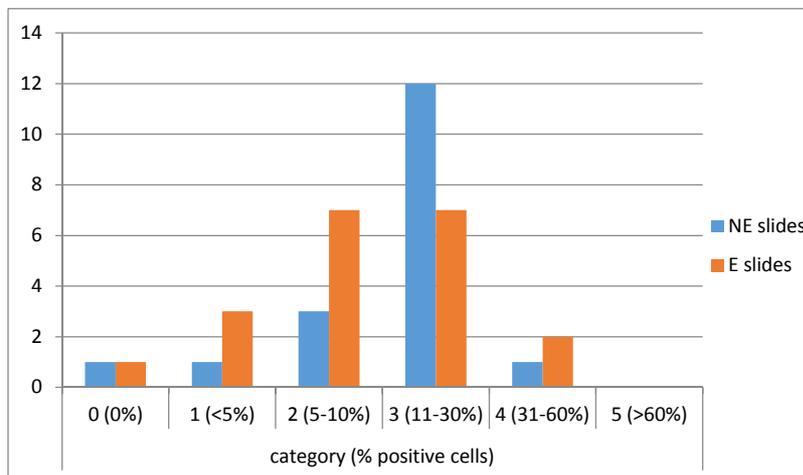


Figure 7c: Non-enlarged adenomas (NE) vs. enlarged adenomas (E)

Mann-Whitney U test statistic: 141.5
p-value: 0.224

Expression of Pax7 and Sox2 in pituitary corticotroph adenomas in dogs

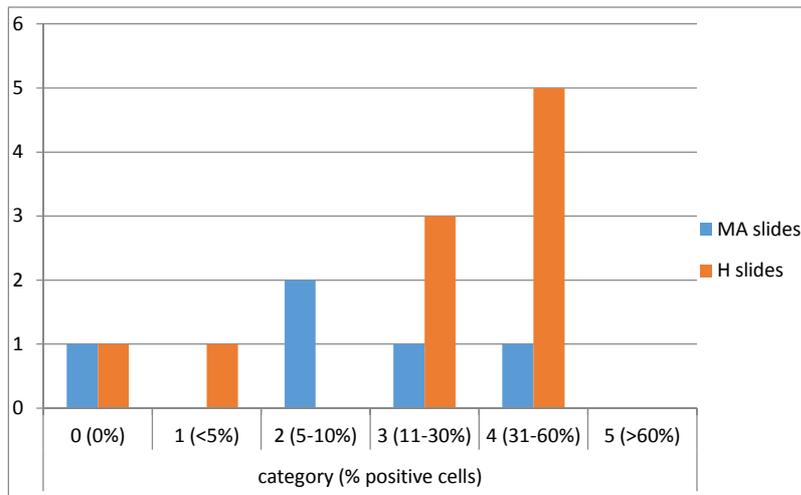


Figure 7d: Malignant tumors (MA) vs. healthy pituitaries (H)

Mann-Whitney U test statistic:
15.5
p-value: 0.224

Table 4 – Statistical analysis of expression of Pax 7 and Sox 2 in different tissue groups.

The Mann-Whitney U test was performed for comparing Sox2- and Pax7-expression of different tissue groups. P-values for all comparisons are displayed below. Significant differences ($p < 0.05$) are displayed in bold letters.

	Pax7	Sox2
NE vs. E	0.007	0.224
NE vs. MA	0.863	0.456
NE vs. ILA	0.266	0.401
NE vs. H	0.003	0.088
E vs. MA	0.070	0.943
E vs. ILA	0.553	0.954
E vs. H	0.210	0.059
MA vs. ILA	0.431	0.932
MA vs. H	0.036	0.224
ILA vs. H	0.161	0.156
ILA vs. NILA	0.724	-

NE: Non-enlarged adenomas; E: Enlarged adenomas; MA: Malignant tumors; ILA: Intermediate lobe adenomas; H: Healthy tissues

Discussion

In search for cancer stem cells that are involved in pituitary adenomas, several markers have been identified, which play an important role in pituitary development, among which Pax7 and Sox2 (Hosoyama, 2010; Budry, 2012; Menks, 2011; Alatzoglou, 2009; Kelberman, 2008). Both Pax7 and Sox2 are transcription factors that are expressed in pituitary progenitor cells. By analyzing the expression of such markers, our understanding about the role of progenitor-/stem- cells in pituitary tumorigenesis can be improved and that can be a starting point for the development of new treatment possibilities. In this study, the expression of Sox2 and Pax7 in pituitary adenomas was analyzed with immunohistochemistry.

No significant differences in expression of Sox2 were found when comparing all different groups, using the Mann-Whitney U test.

For Pax7, an expected difference was that IL-adenomas express more Pax7 than healthy tissue, but this was not the case. However, significant differences have been found for three other comparisons: the comparison between non-enlarged adenomas and enlarged adenomas; the comparison between healthy tissues and non-enlarged adenomas; and the comparison between healthy tissues and tumors with malignant characteristics. In contrast with our hypothesis, healthy tissues expressed more Pax7 than non-enlarged adenomas and malignant tumors. It is difficult to find an explanation for this. It is remarkable that for both Pax7 and Sox2, the healthy tissues show a relatively high expression. A possible conclusion could be that healthy tissue actually is more positive for these markers than tumor tissue. However, this high expression may also be due to a relatively good quality of the healthy tissue samples that were used. The healthy tissue samples sometimes seemed to be more intact, and more complete. Maybe these slides coincidentally included the parts with most positive cells. The finding that enlarged adenomas express more Pax7 than non-enlarged adenomas is interesting however, because the overall hypothesis of this research project was that enlarged tumors would express more Pax7 and/or Sox2 than non-enlarged tumors.

No correlations between pax7- and Sox2-expression in the various groups and survival and disease free intervals have been examined yet, so no conclusions about effects of Pax7- and Sox2-expression on survival can be drawn. This will be done in a later phase of this research project.

The results presented here are partly dependent on what tissue samples were collected during surgery, as well as which paraffin blocks were available and used for immunohistochemistry. Even though it has been made sure that the right paraffin block (with tumor tissue included) was collected, it could sometimes be that, accidentally, the tissue with the most positive cells was not included in any paraffin block and so has not been used for this study. Indeed, in several samples only (small) parts of the pituitary tissue were present (and not the whole pituitary). So maybe a certain pituitary certainly contained a lot of positive cells, but they were just not observed in immunohistochemistry because the part with the most positive cells was missing in the slide.

The results also partly depend on which slices have been made of a paraffin block. Maybe another part of the paraffin block (on another level) would have been much more representative for the whole pituitary tissue.

Furthermore, there could be small differences in execution of the IHC protocols and conditions in which the protocols are executed. By randomizing the slides with tumor tissue during IHC, any differences in protocol-execution between the different pituitary groups were ruled out for a large part. The slides with healthy tissue however, were not randomized together with the 50 tumor tissue-slides, but this was controlled for by taking along also one slide (one for Pax7 and one for Sox2) from the group of healthy tissues in each batch of tumor tissue-slides.

As mentioned in the introduction, according to the study of Budry et al (2012), in dogs approximately 30% of pituitary corticotroph adenomas express Pax7. In this study, however, that proportion is much higher: of the total of 50 tumor tissues, 40 tissues expressed Pax7 (80%). It is difficult, however, to interpret this difference, because of potential differences in methods of assessment of Pax7- expression.

Also, the absence of Pax7-expression in a slide does not mean the whole tumor tissue was really negative for Pax7; maybe not the whole tumor tissue is included in the slide.

For interpreting the expression of Pax7 and Sox2 in the tumor tissues, it is important to know what kind of cells in a slide are positive; the tumor cells or the healthy/normal cells. In many cases, a slide does not only contain tumor tissue but also normal tissue. For identifying the actual tumor tissue in the slides, H&E slides should have been made to compare the immunostained slides with. This was however not the case. Now, only a rough assessment could be done with the help of a pathologist (Dr. G. Grinwis) on what tissue represented the tumor tissue. So, it is not always clear if it's mainly the tumor cells or the normal cells that stained positive for Pax7 and Sox2. When this research project is continued, H&E slides should be made and used for identifying tumor localization.

As mentioned before, different Pax7 populations were observed. In many slides, with both healthy and tumor tissue, (large or small) clusters of positive cells (sometimes clearly in the IL) were seen, which were mostly dark brown, but also cells scattered through the tissue which were mostly lighter brown. Maybe these populations represent a different kind of cells.

A possible theory could be that the lighter brown cells are descendants of the darker brown cells, and therefore do not express as much Pax7 anymore as the dark brown cells do. This may also be the reason for the fact that the lighter brown cells are mostly scattered through the (probably adenohipophyseal) tissue and are not clustered in one spot. This finding could be in line with the earlier mentioned theory of Hosoyama et al (2010), which implied that during differentiation, one proportion of Pax7+ cells migrates through the IL to eventually differentiate to melanotrophs and another proportion of Pax7+ cells migrates to the AL to differentiate to other endocrine cells. However, from findings in the other studies on Pax7, it was not expected that Pax7-positive cells would be observed in the AL; this has not been reported before (Budry, 2012).

There is one study that shows that Sox2 can also be present in the cytoplasm of embryonic stem cells (Gretchen et al, 2009). This was, however, not seen in the (adult) pituitary tissues that were used in this research project.

Some Sox2-slides contained very light brown cells that could almost not be recognized as positive cells. When this is the case, it is more difficult to estimate the percentage of positive cells in a slide. Therefore, it can be questioned if the chosen antibody concentration for Sox2 (1:800) was strong enough. This antibody concentration was chosen because in this way, the different intensities of Sox2 expression in cells could be visualized. But eventually, differences in intensity between cells were not really further analyzed in this report, so it was probably not really necessary to use this relatively low antibody concentration.

In several slides, Sox2-positive cells have also been found in the (stroma of the) neurohypophysis. This is not documented in other studies on Sox2-expression in the pituitary, but it can be explained by the fact that Sox2-expression is also observed in neural tissues.

Since Budry et al (2012) showed expression of both Pax7 and Sox2 in some cells in the IL, it would be interesting to examine if also in the tissues used in this study, cells expressing both markers are present.

The scoring of the slides could have been done in a somewhat more reliable manner, by reaching an agreement on the scores of the 49 slides that were scored differently by the two different persons that scored the slides.

In order to do this, the two persons that scored the slides could have looked together at the slides that were scored differently and come to an agreement, or otherwise, a third person could have scored them as well. However, in this project, there was no time left to score the slides in such an extensive manner, so finally for the slides that were scored differently, only the scores of one person were used.

Actually the procedures used for scoring the expression of Pax7 and Sox2 were not very exact; we estimated the total percentage of positive cells in a slide. Calculating the labeling indices would be much more precise, but in this research project there was no time to count the positive cells in all the slides. This could maybe explain the fact that not all the results turned out the way that was expected. Maybe different results would be found when labeling indices were used instead of estimated percentages.

In conclusion, in general, no evident differences were observed when comparing different tumor tissues and healthy tissues for Pax7- and Sox2-expression. Significant differences were found for Pax7 expression for three comparisons, but only one was consistent with the hypothesis (that enlarged adenomas express more Pax7 than non-enlarged adenomas). The two other significant differences were not expected; healthy tissues showed higher expression than the non-enlarged adenomas and the malignant tumors. It was remarkable that a larger proportion of tissues from IL-adenomas contained clusters with Pax7-positive cells (71%) than healthy tissues (40%).

More research is necessary to further investigate the expression of these markers in pituitary tumor tissues. It is recommended to score the slides more accurately (see above) and also to perform statistical analyses more extensively. In this manner, maybe different results are found. More research should also be done on the role of these markers in pituitary tumorigenesis and tumor progression.

For further research, correlations between Pax7- and Sox2-expression and disease free intervals and survival rates should be examined. In this way, the clinical significance of Pax7- and Sox2- expression in pituitary adenomas, and the usefulness of these markers for the development of new therapeutic interventions for Cushing's disease becomes clear.

References

- Alatzoglou, K.S. et al. (2009) The role of SOX proteins in normal pituitary development. *Journal of endocrinology* **200**, 245-258
- Budry, L. et al. (2012) The selector gene Pax7 dictates alternate pituitary cell fates through its pioneer action on chromatin remodeling. *Genes Dev.* 26: 2299-2310
- Chen, Y. et al. (2008) The molecular mechanism governing the oncogenic potential of SOX2 in breast cancer. *Journal of Biological Chemistry*, vol. 283, no. 26, pp. 17969-17978
- Dong, C., Wilhelm, D. & Koopman, P. (2004) Sox genes and cancer. *Cytogenetic and Genome Research*, vol. 105, no. 2-4, pp. 442-447
- Gleiberman, A.S. et al. (2008) Genetic approaches identify adult pituitary stem cells. *PNAS*; vol. 105, no. 17, 6332-6337
- Gretchen, H. et al. (2009) Acetylation of Sox2 Induces Its Nuclear Export in Embryonic Stem Cells. *Stem cells express* 27: 2175–2184
- Hanson, J.M. et al. (2005) Efficacy of Transsphenoidal Hypophysectomy in Treatment of Dogs with Pituitary-Dependent Hyperadrenocorticism. *J Vet Intern Med* 19: 687–694
- Hanson, J.M. et al. (2007) Prognostic factors for outcome after transsphenoidal hypophysectomy in dogs with pituitary-dependent hyperadrenocorticism. *J Neurosurg* 107: 830–840
- Hill, R.E. et al. (1991) Mouse small eye results from mutations in a paired-like homeobox-containing gene. *Nature*, vol. 354, no. 6354, 522-525
- Hosoyama, T. et al. (2010) A postnatal Pax7+ progenitor gives rise to pituitary adenomas. *Genes and Cancer* 1(4) 388-402
- Kelberman, D. et al. (2008) SOX2 plays a critical role in the pituitary, forebrain, and eye during human embryonic development. *J Clin Endocrinol Metab.* 93(5): 1865-1873
- Kooistra H.S., Galac, S. (2012) Recent Advances in the Diagnosis of Cushing's Syndrome in Dogs. *Topics in Companion Animal Medicine* 27: 21-24
- Kuroda, T. et al. (2005) Octamer and Sox Elements Are Required for Transcriptional *cis* Regulation of *Nanog* Gene Expression. *Mol. Cell. Biol.* vol. 25, no. 6, pp. 2475-2485
- Melmed, S. (2011) Pathogenesis of pituitary tumors. *Nat. Rev. Endocrinol.* **7**, 257-266
- Menks, K.C. (2011) The role of transcription factors Sox2 and Sox9 in the forming of pituitary gland adenomas in dogs. Bachelor thesis.
- Nassiri, F. et al. (2013) Pituitary stem cells: candidates and implications. *Pituitary*

van Rijn, S.J. et al. (2010) Expression of Ki-67, PCNA, and p27kip1 in canine pituitary corticotroph adenomas. *Domestic Animal Endocrinology* 38: 244–252

Nolan, L.A., Levy, A. (2006) A Population of Non-Luteinising Hormone/Non-Adrenocorticotrophic Hormone-Positive Cells in the Male Rat Anterior Pituitary Responds Mitotically to Both Gonadectomy and Adrenalectomy. *Journal of Neuroendocrinology* 18, 655–661

Nolan, L.A., Levy, A. (2009) The Trophic Effects of Oestrogen on Male Rat Anterior Pituitary Lactotrophs. *Journal of Neuroendocrinology* 21, 457–464

Phi, J.H. et al. (2008) Sox2 expression in brain tumors: A reflection of the neuroglial differentiation pathway. *American Journal of Surgical Pathology*, vol. 32, no. 1, pp. 103-112

Rijnberk, A., Kooistra, H.S. (2010) *Clinical Endocrinology of Dogs and Cats. An Illustrated Text. Second, revised and extended edition.*

Seale, P. et al. (2000) Pax7 is required for the specification of myogenic satellite cells. *Cell*, vol. 102, no. 6, 777-786

Vankelecom, H., Gremeaux, L. (2010) Stem cells in the pituitary gland: A burgeoning field. *General and comparative Endocrinology* 166, 478-488

Appendix 1

Pax7 immunohistochemistry protocol

Day 1

1. Deparaffinization:
 - a. 5 min. Xylene 1
 - b. 5 min. Xylene 2
 - c. 5 min. Ethanol 96%
 - d. 5 min. Ethanol 80%
 - e. 5 min. Ethanol 70%
 - f. 5 min. Ethanol 60%
 - g. 5 min. PBS
2. Antigen unmasking:
 - a. Place a bin with 10 mM Citrate with 0.05% Tween 20 (pH 6) in a water bath at 98 °C, during deparaffinization. Then place the slides in the Citrate bin in the water bath for 1 hour.
 - b. Cool down for 20 minutes at room temperature (place Citrate bin with slides outside water bath)
3. Put slides in PBS for 5 minutes; dry slides with a tissue and encircle tissue with marker; put slides in PBS again for 5 minutes.
4. Blocking:
 - a. Drop 1 – 3 drops of Peroxidase (Endogenous enzyme block) on the tissue; leave it for 5 minutes; then let it flow off.
 - b. Put slides in PBS, then dry slides with a tissue.
 - c. Mix Normal goat serum and Antibody diluent in the ratio of 1:10 on a vortex and pipette about 50 µl of this mixture on each slide, until the tissue is fully covered.
 - d. Incubate the slides for 30 minutes in a covered box with wet tissues at the bottom.
5. Primary antibody (Mouse):
 - a. Dry slides with a tissue
 - b. Pipette about 50 µl of the diluted Pax7 antibody solution (antibody concentration of 1:20) on each slide, until it's fully covered. Except for the negative control slide, which must be covered with 50 µl of Antibody diluent.
 - c. Incubate the slides in a covered box with wet tissues at the bottom, over night at 4°C.

Day 2

6. Washing:
 - a. 3 x 5 min. in PBS on mechanical shaker
7. Secondary antibody (Anti-mouse):
 - a. Dry slides with a tissue

- b. Drop 1 – 3 drops of the secondary antibody solution on each slide, until tissue is fully covered.
 - c. Incubate the slides for 30 minutes in a covered box with wet tissues at the bottom.
8. Washing:
 - a. 3 x 5 min. in PBS on mechanical shaker
9. Colouring with DAB:
 - a. Dry slides with a tissue
 - b. Mix 1 ml of DAB substrate with 1 drop of DAB chromogen on a vortex and pipette about 50 µl of the solution on each slide, until it's fully covered.
 - c. Keep track of time and let the DAB flow off when the tissue's coloured brown enough.
10. Put the slides in PBS for 5 minutes
11. Dyeing with Hematoxyline:
 - a. Put 1 – 3 drops of Hematoxyline on each slide for 10 seconds, then let it flow off.
 - b. Put the slides in H₂O
12. Place the waterbin with the slides under a running tap for 10 minutes (aim the tap on the rim of the bin).
13. Dehydration:
 - a. 5 min. 60% Ethanol
 - b. 5 min. 70% Ethanol
 - c. 5 min. 80% Ethanol
 - d. 5 min. 96% Ethanol 1
 - e. 5 min. 96% Ethanol 2
 - f. 5 min. Xylene 1
 - g. 5 min. Xylene 2
14. Vectamount:
 - a. Put 1 drop of Vectamount on each slide (after letting it dry to the air for some seconds) and cover it with a coverslip. Remove air bubbles if necessary.

Sox2 immunohistochemistry protocol

Day 1

1. Deparaffinization:
 - a. 5 min. Xylene 1
 - b. 5 min. Xylene 2
 - c. 5 min. Ethanol 96%
 - d. 5 min. Ethanol 80%
 - e. 5 min. Ethanol 70%
 - f. 5 min. Ethanol 60%
 - g. 5 min. PBS
2. Antigen unmasking:

- a. Place a bin with 10 mM Citrate with 0.05% Tween 20 (pH 6) in a water bath at 98 °C, during deparaffinization. Then place the slides in the Citrate bin in the water bath for 30 minutes.
- b. Cool down for 20 minutes at room temperature (place Citrate bin with slides outside water bath)
3. Put slides in PBS for 5 minutes; dry slides with a tissue and encircle tissue with marker; put slides in PBS again for 5 minutes.
4. Blocking:
 - a. Drop 1 – 3 drops of Peroxidase (Endogenous enzyme block) on the tissue; leave it for 5 minutes; then let it flow off.
 - b. Put slides in PBS, then dry slides with a tissue.
 - c. Mix Normal goat serum and Antibody diluent in the ratio of 1:10 on a vortex and pipette about 50 µl of this mixture on each slide, until the tissue is fully covered.
 - d. Incubate the slides for 30 minutes in a covered box with wet tissues at the bottom.
5. Primary antibody (Rabbit):
 - a. Dry slides with a tissue
 - b. Pipette about 50 µl of the diluted Pax7 antibody solution (antibody concentration of 1:800) on each slide, until it's fully covered. Except for the negative control slide, which must be covered with 50 µl of Antibody diluent.
 - c. Incubate the slides in a covered box with wet tissues at the bottom, over night at 4°C.

Day 2

6. Washing:
 - a. 3 x 5 min. in PBS on mechanical shaker
7. Secondary antibody (Anti-rabbit):
 - a. Dry slides with a tissue
 - b. Drop 1 – 3 drops of the secondary antibody solution on each slide, until tissue is fully covered.
 - c. Incubate the slides for 30 minutes in a covered box with wet tissues at the bottom.
8. Washing:
 - a. 3 x 5 min. in PBS on mechanical shaker
9. Colouring with DAB:
 - a. Dry slides with a tissue
 - b. Mix 1 ml of DAB substrate with 1 drop of DAB chromogen on a vortex and pipette about 50 µl of the solution on each slide, until it's fully covered.
 - c. Keep track of time and let the DAB flow off when the tissue's coloured brown enough.
10. Put the slides in PBS for 5 minutes
11. Dyeing with Hematoxyline:
 - a. Put 1 – 3 drops of Hematoxyline on each slide for 10 seconds, then let it flow off.
 - b. Put the slides in H₂O

12. Place the waterbin with the slides under a running tap for 10 minutes (aim the tap on the rim of the bin).
13. Dehydration:
 - a. 5 min. 60% Ethanol
 - b. 5 min. 70% Ethanol
 - c. 5 min. 80% Ethanol
 - d. 5 min. 96% Ethanol 1
 - e. 5 min. 96% Ethanol 2
 - f. 5 min. Xylene 1
 - g. 5 min. Xylene 2
14. Vectamount:
 - a. Put 1 drop of Vectamount on each slide (after letting it dry to the air for some seconds) and cover it with a coverslip. Remove air bubbles if necessary.