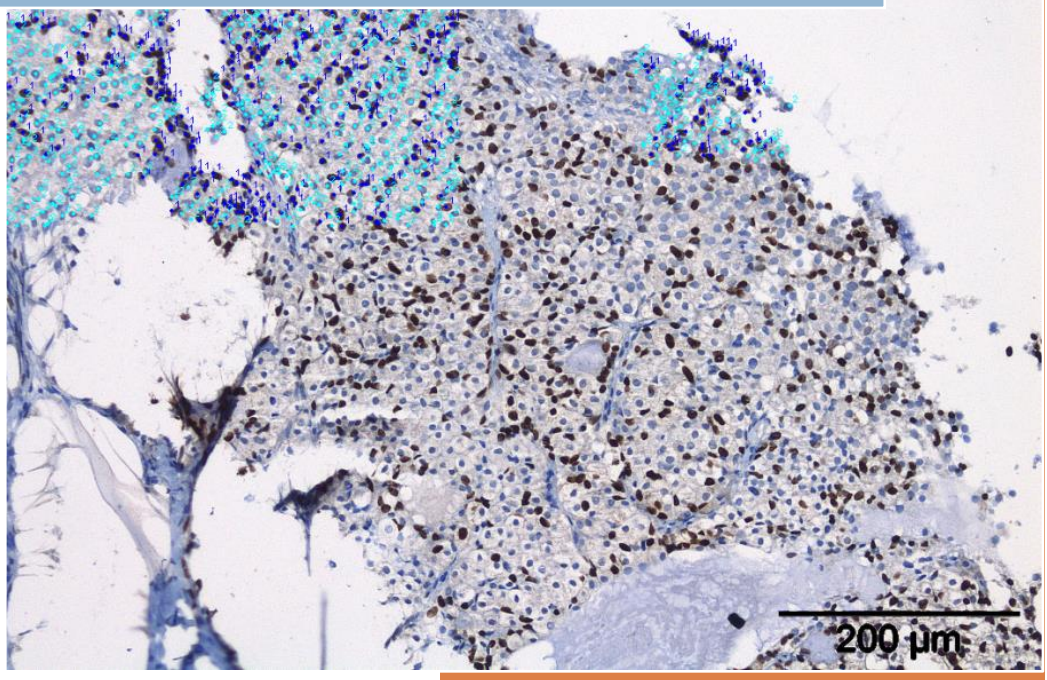
Expression analysis of Pax7 and Sox2 in canine pituitary gland adenomas and correlation with clinical parameters



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Abstract

Cushing's disease or pituitary-dependent hypercortisolism(PDH) is a common endocrinologic disorder in dogs. In 80% to 85% of cases of hypercortisolism, the cause is a pituitary gland adenoma. The pituitary adenoma mostly arises from the adenohypophysis (75% to 80%)and in the other cases from the pars intermedia. The diagnosis of Cushing's disease is based on urinary cortisol/creatinine ratios (UCCRs) combined with a high dose dexamethasone suppression test (HDDST).

Without treatment the life expectancy of dogs with Cushing's disease is less than one year.[1] There are different therapeutic options for dogs with Cushing's disease.

Medical treatment is possible with Mitotane or Trilostane. Also surgery is a treatment option, than the complete pituitary gland is removed by transsphenoidal hypophysectomy. However, even after initial remission of the disease, long- term recurrence does occur. The pathogenesis of pituitary gland adenomas is largely unknown. It is thought that so called cancer stem cells play a role in tumorigenesis. When such cells can be identified, new therapeutic strategies, directed at these stem cells, could be developed. To identify potential cancer stem cells in the pituitary gland, certain markers can be used. Transcription factors Sox2 and Pax7 are possible stem cell marker. They are both transcription factors that are thought to play a role in pituitary development and tumorigenesis.

In this research project the expression of the markers Pax7 and Sox2 in pituitary gland adenomas was investigated. Positive Pax7 and Sox2 cells are counted with ImageJ and the labeling indices were calculated. The expression was correlated with clinical parameters such as, tumor type, the size of the pituitary gland, disease free interval of the dog, survival time of the dog and recurrence of Cushing's disease after surgery. No significant difference was found when the expression of Pax7 and Sox2 is compared between different groups of clinical parameters, like non-enlarged and enlarged. However, the survival time of the patients shows that there was a positive correlation between the expression of Sox2 and the survival time. No significant difference was found in the survival analysis when the survival time of Pax7 positive patients and Pax7 negative patients were compared and when patients with low Sox2 expression and patients with high Sox2 expression were compared.

In conclusion, more research is necessarily to investigate if there is a relation between the expression of these markers and their role in tumorigenesis and their relation with clinical parameters.

Introduction

The pituitary gland

The pituitary gland is an endocrine gland located at the base of the brain. It consists of three parts: the largest part is the adenohypophysis (anterior lobe), a smaller part is formed by the neurohypophysis (posterior lobe) and the pars intermedia.[1] The adenohypophysis and neurohypophysis develop from an interaction between the ectoderm of the dorsal aspect of the primitive mouth and the ventral neuroectoderm of the diencephalon.[10,22] There is an outgrowth of the roof of the primitive mouth which forms Rathke's pouch. The anterior wall of Rathke's pouch proliferates and develops the adenohypophysis. Also the diencephalon thickens and form the infundibulum that later expands and develops into the neurohypophysis.[10,23] The space between both lobes is called the hypophysial cleft. The tissue caudal of this is called the pars intermedia.(Figure 1) It also develops from Rathke's pouch and it's directly applied to the posterior lobe.[1,2] The adenohypophisis surrounds the neurohypophysis. The adenohypophysis contains five hormone producings cell types, that all produce specific hormones. Cells expressing POMC lead to the secretion of adrenocorticotrophic hormone. Gonadotroph cells secrete follicle stimulating hormone (FSH) and luteinizing hormone (LH). Pit1- dependent cells lines (somatotroph, lactotroph and thyrotroph cells) lead to the secretion of growth hormone (GH), prolactin and thyroid stimulating hormone (TSH). The pars intermedia contains cells that express pro- opiomelancortin (POMC) that leads to the secretion of α melanocyte stimulating hormone (α -MSH) .[1,2] The neurohypophysis excretes oxytocin, which has an effect on the smooth muscles of the uterus and the myoepthial cells of the udder, and vasopressin which has an effect on the kidneys.[2]

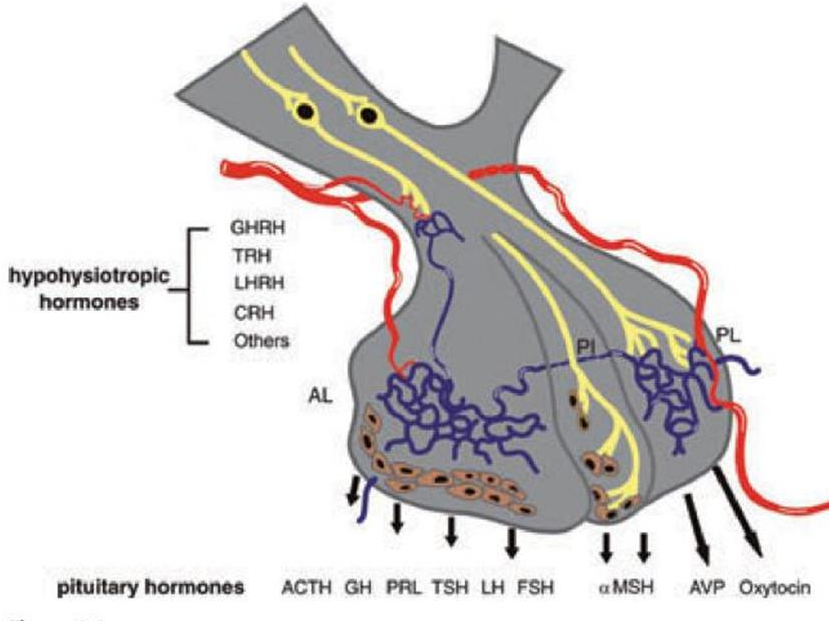


Figure 1. The pituitary gland and the hormones secretion
(Rijnberk A. Kooistra HS. Clinical Endocrinology of Dogs and Cats, 2010)

Cushing's disease

Cushing's disease or pituitary-dependent hypercortisolism (PDH) is an endocrinologic disorder. It's a common disorder in dogs and a rare disorder in cats.[3] In dogs there is no sex predilection and it occurs in all dog breeds, however it seems to occur a slightly more in small breed dogs. The dogs are often middle aged or older aged, it also appears in dogs as young as one year but this is rare.[1] Hypercortisolism is caused by a pituitary adenoma of the corticotroph cells, that produces adrenocorticotropin hormone (ACTH).[3] In 80% to 85% of cases of hypercortisolism the cause is a pituitary gland adenoma and we talk about PDH. The other 15% to 20% is caused by an adrenocortical adenoma or adenonocarcinoma.[4] The pituitary adenoma mostly arises from the adenohypophysis (75% till 80%) and in the other cases from the pars intermedia.[5] It can also occur in both lobes. It both causes PDH because both lobes have cells that synthesize POMC which can be converted into ACTH and then into α- MSH. This is clinically interesting because tumors of the pars intermedia tend to be larger than the ones of the anterior lob. [1]
When there is a corticotroph tumor in the anterior lob or pars intermedia, this produces more ACTH than usual resulting in a hypersecretion of cortisol and thus hypercortisolism, because the high plasma of cortisol has no influence on the pituitary gland adenoma and the production of ACTH will not be inhibited.[1,4]
Common clinical signs of Cushing's disease are polyuria, polyphagia, thin hair coat, enlarged abdomen, obesity, high plasma concentrations of cortisol and atrophy of the muscles and skin (Figure 2). When the pituitary tumor is large, it can also cause neurologic signs. [1,4]

Diagnosis of Cushing´s disease

The diagnosis of Cushing's disease is based on urinary cortisol/creatinine ratios (UCCRs) combined with a high dose dexamethasone suppression test (HDDST). First, urinary samples of the dog must be collected. The owner can accomplish this and it's important that the samples are collected under conditions free of stress. The excess secretion of cortisol can be measured in the urine by determination of the urinary cortisol/creatinine ratios (UCCRs). The diagnosis hypercortisolism is confirmed based on elevation of UCCRs in the first two samples. The next step is to perform a high dose dexamethasone suppression test. Three oral doses of dexamethasone 0,1 mg per kg body weight are given at 8 hours intervals. Then a third urinary sample must be collected. If in this third sample the UCCR is decreased more than 50% from baseline values the diagnosis of PDH is confirmed. [4,6]
However, when there is less than 50% suppression, PDH is still possible. This can be caused by a cortisol resistant anterior lobe or an intermediate lobe adenoma. The high plasma of cortisol has no influence on the pars intermedia, because the pars intermedia is under direct neural control, principally tonic dopaminergic inhibition, which supresses the expression of glucocorticoid receptors. [4] When PDH is confirmed computed tomography(CT) or magnetic resonance imaging (MRI) is performed to visualise the tumor in the pituitary gland and to determine pituitary size. Also CT-scan allows assessment of surgical landmarks needed for transsphenoidal hypophysectomy.[4]

Figure 2. (left)An affenpischer with Cushing's disease (right) after Hypophysectomy ***(Rijnberk A. Kooistra HS. Clinical Endocrinology of Dogs and Cats, 2010)***

Treatment of Cushing's disease

Without treatment the life expectancy of dogs with Cushing's disease is less than one year.[1] There are different therapeutic options for dogs with Cushing's disease. Treatment at adrenal level can be done when the tumor is not that expansive that it causes neurologic problems, but it doesn't take away the cause of the disease, which is the pituitary adenoma.

Mitotane is a drug that can be used for medical management of PDH. It works at adrenal level and causes a selective destruction of the adrenal cortex.[3,7] The treatment occurs in two phases. The first phase is the loading phase so rapid destruction of adrenal tissue occurs. The required dose is 25 to 50 mg/kg per day (5 till 65 days). After this, the dosage is switched to a maintenance dosage, which is lower. The most common side effects of Mitotane are vomiting, diarrhea, anorexia, weakness and ataxia. Relapse is common by this treatment (50%) and a second loading dose is necessary. [7]

Another possibility and currently the most used treatment is Trilostane, that inhibits the 3 beta- hydroxysteroid dehydrogenase/isomera system. This system is essential for the synthesis of cortisol, thus, cortisol secretion is inhibited. [1,7] Trilostane must always be administered with food. Side effects of Trilostane are vomiting, diarrhea, anorexia, shaking, lethargy and inappentence. [7]

Surgical treatment is directed against the pituitary adenoma. The complete pituitary gland can be removed by transsphenoidal hypophysectomy. This therapy is an effective

method to treat dogs with PDH.[3] (Figure 2, right) The recurrence rate is lower with hypophysectomy than with treatment with mitotane.[3] And also the disease free interval and survival time is longer with surgical treatment than treatment with mitotane. [8] However, dogs need life- long hormone suppletion therapy and long- term recurrences do occur in 23% of cases.[19] The prognosis after surgery is better for dogs with non-enlarged pituitaries (P/B ratio<0,31) compared to dogs with enlarged pituitaries.[8]

Pathogenesis of pituitary tumors

The pituitary gland has an important endocrinologic function. The control of this function involves hypothalamic, intrapituitary and peripheral signals. This results in a coordinated regulation of pituitary hormone synthesis and secretion and also in a selective pituitary cell proliferation.[9] There are different lines of cells in the pituitary gland, this enables the expansion of a population of highly specific early pituitary progenitor cells and their progression to terminally differentiated, hormone-expressing pituitary cells.[9]
Expression of T-pit that binds with corticotropin upstream transcription-binding element (CUTE) proteins determines the differentiation of the pro- opiomelanocortin cell lineage, corticothroph and melanotroph cells. Expression of SF-1, GATA-2 and Lhx4 is necessary for differentiation of gonadotroph cells. Development of somatotroph, mamosomatotroph, lactotroph and thyrotroph cells is possible by expression of Pit-1 and Prop-1.[23]

Postnatal and during life, the composition of cells in the pituitary gland can change. For example during growth, stress, puberty, pregnancy and the estrous cycle. The regulation of pituitary cell proliferation is done by humoral factor and intrapituitary signaling.
An example is during pregnancy when the level of lactotrophs cells mostly increase, due to an elevated estrogen level.[9]

Pituitary adenomas are monoclonal but are composed of various cell types, which suggest that they arise from expansion of single precursor cells that may give rise to pituitary adenomas. [9] It is thought that cancer stem cells play a role in pituitary tumorigenesis. Stem cells are cells that have the ability for self-renewal and they can differentiate into multiple lineages. Stem cells are not associated with one cell type, different cell types are indicated as stem cells, such as folliculostellate cells, side population (SP) cells, GFRa2/Prop1-positive stem (GPS) cells, marginal cells, Nestin-expressing cells or cells that express Sox2. [10, 11] These cells all have the same characteristics, such as their location along the Rathke's cleft, their ability to form spheres in culture and to differentiate into the five hormonal cell lineages.[10]
CSCs are cells that have the ability to drive the growth, composition, invasion, metastasis of a tumor. [10,12]

Not much research in dogs is done into the role of stem cells in pituitary tumorigenesis. Several studies are done in humans and animals (mostly mice) to search for stem cell markers. Nestin positive cells are already identified as cells that play a part in the tumorigenenis in α-MSH adenomas.[11] Another study has shown that Pax7 expressing cells play a part in the pituitary tumorigenesis.[13] Also Sox2 expressing cells seems to have a role in the tumorigenesis. [12] When CSCs can be identified, new therapeutic strategies, directed at these stem cells, could be developed.

Function of Pax7 and its role in tumorigenesis

Pax7 is a transcription factor that is important in embryonic patterning and postnatal stem cell renewal in many organs, such as the eye, brain and muscles.[5] Pax7 plays a critical role in maintaining tissue specific stem cell populations in the muscles. It seems also that Pax7 plays a part in the postnatal pituitary growth.[5]

Pax7 expressing cells in mice are localized in the intermediate lobe and in the lumen between the intermediate lobe and the anterior lobe. [13] Its thought that Pax7 expressing cells are endocrine cells given to their location in the IL. The cells in the IL are melanotrophs and they express Pax7. Pax7 is a critical positive regulator of the melanotrope fate. Because it acts as a selector switch between the POMC lineages for melanotrope cells and corticotrophs cells. It's critical for activation of melanotrope-specific genes and repression of corticotrope-specific genes. It isn't critical for the differentiation of melantrophs because also Tpit is necessary, Pax7 only increases the chromatin accessibility of the regulatory sequences for Tpit.[14]

According to a study of Hoyosama et al, Pax7 can give rise to silent corticotroph macroadenomas when the tumor suppressor gene RB was removed in mice. The tumor also express α-MSH, which is made of ACTH. This is characteristic for a melanotroph lineage tumor.[13] Also it's investigated that 30% of the canine pituitary gland adenomas express Pax7.[14] This can be interesting for the correlation between clinical signs and the level of Pax7 expressing cells. This can be important for the development of therapeutic strategies, regarding to melanotrope specific functions because of the regulatory role of Pax7 in the melanotroph lineage.[14]

Function of Sox2 and its role in tumorigenesis

Sox genes are transcription factors which belong to the High Mobility Group(HMG) superfamily. There are different species and they play a part in different developmental processes like pluripotency and determination of cell fate to neurogenesis sex development, chrondrogenesis, haematopoiesis.[15,16]

Sox2 is a transcription factor which is expressed in several organs like the stomach, testes, lens and the pituitary gland. It plays a crucial role in the development of the central nervous system. Sox2 plays an important role in the development of the pituitary gland. [17] During pituitary development Sox2 is expressed in Rathke's pouch and also is detected in proliferating cells of the dorsal zone and in scattered cells of the anterior pituitary.
In the adult pituitary gland there is expression of Sox2 in cells lining the pituitary cleft or scattered in the parenchyma, so that maintain their potential to differentiate into all pituitary cell types and act like progenitor cells. This persistence may be crucial for the plasticity and dynamic response of the gland to fluctuating endocrine demands and its capacity to regenerate after trauma.[15,16] Sox2+ cells could possibly be a stem cell population, because they divide slowly, express stem cell antigen-1 in mice, differentiate

and don't produce hormones and are able to generate secondary pituispheres. However, further research must be done to determine this.[11]
It's already investigated that Sox2 is expressed in brain tumors. So it's possible that Sox2 is also expressed in pituitary tumors.[18] During a study in mice, it was investigated that Sox2 adult mouse pituitary cells can differentiate and self- renew in vitro. But their role in pituitary tumorigenesis remains still unknown. [17] So more research is needed to find out if Sox2 plays a role in pituitary tumorigenesis.

Aim of this study

In this research project the expression of the markers Pax7 and Sox2 in pituitary gland adenomas will be investigated. The expression will be correlated with clinical parameters such as, tumor type, the size of the pituitary gland, disease free interval of the dog, survival time of the dog and recurrence of the tumor after surgery. This can give more knowledge about the usefulness of these markers in giving prognosis and prediction of outcome in dogs with a pituitary adenoma. The hypothesis of this research project is when there's a high expression of Pax7 and Sox2 in pituitary gland adenomas, the prognosis for the dog is less favorable.

Materials and Methods

Animals

In this study, samples of 44 pituitary adenomas from client owned dogs (table 1), all diagnosed with Cushing's disease, and of 6 pituitary glands of healthy laboratory dogs (table 2) were used. All client owned dogs had transsphenoidal hypophysectomy as first treatment, which was performed in the University clinic in Utrecht. The adenoma samples were collected during pituitary surgery. The normal pituitary samples were collected at termination of other, non- related, DEC approved experiments.
Clinical, biochemical, imaging and hormonal information of all dogs were available in the medical records in Vetware and from the database of Dr. B. Meij. When information wasn't complete this is updated by calling the owner of the dog. Patient selection was based on clinical parameters, such as histological examination and pituitary size, expressed in the P/B ratio.
The P/B ratio is the pituitary-height/brain-area ratio, with this ratio the difference can be made between enlarged and non- enlarged pituitary adenomas. Enlarged pituitaries have a P/B ratio higher than 0.31 and non-enlarged pituitaries have a ratio less than or equal to 0.31.[19] The follow up and the disease free interval is updated for all patients by calling the owners of the patients. Remission by the patients was defined when UCCR lower was than 10 x 10-6 and when resolution of clinical signs of hyperadrenocorticism was defined. Recurrence by the patients was defined when UCCR was equal or higher than 10 x 10-6 and when return of clinical signs and symptoms of hyperadrenocorticism after initial remission was defined.[19] Clinical information about the patients is depicted in Table 1.

Table 1. Clinical information of the patients

No.	Age (y)	Sex	Weight (kg)	Breed	C/C12	% suppression	Pituitary size (HxWxL)	P/B ratio
1	5,2	M	38	American Staffordshire Terrier	69,0	93,3	5,6 x 7,2 x 6	0,29
2	9,4	M	8,35	Crossbreed	32	96,9	3,2 x 4 x 4	0,23
3	7,9	M	8	Bolognese	64,5	96,9	3 x 4,2 x 4	0,20
4	6,1	F	50	Dogue de Bordeaux	36	91,4	6 x9 x 10	0,26
5	6,7	M	8,2	Yorkshire Terrier	42	96,7	4 x 4 x 4	0,24
6	3,9	M	22,8	English Cocker Spaniel	64,5	-58,1	15 x 17 x 18	0,76
7	10,1	M	25,2	Crossbreed	57,0	94,4	11,5 x 10,6 x 10	0,70
8	8,6	M	33,4	Boxer	44,9	92,4	12,4 x 11, 6 x 11	0,70
9	5,8	F	16,7	English Cocker Spaniel	96,1	69,4	14,2 x 17,4 x 17	1,10
10	9,4	F	19,5	German Shorthaired Pointer	59,0	92,4	7,4x 8,6 x 8	0,45
11	6,1	M	48,2	Golden Retriever	13,2	87,1	18,3 x 19,6 x 17,2	0,95
12	11,9	F	23,0	German Wirehaired Pointer	81,5	14,1	7,6 x 8,7 x 11,4	0,47
13	11,7	M	13,0	Jack Russell Terrier	13,5	74,1	12,6 x 13,4 x 16, 2	0,74
14	12,1	M	24.0	Beagle	N/A	N/A	22,5 x 21,3 x 20,7	1,25
15	7,3	M	23,0	Crossbreed	0,0	N/A	10,4 x 11 x 11, 3	0,65
16	8,3	F	36,5	Labrador Retriever	30,7	88,3	11,5 x 11,8 x 11, 6	0,56
17	8,2	M	23,2	Beagle	74,5	75,8	16,6 x 14,6 x 16	1
18	6,5	M	12,8	Crossbreed	81,0	60,5	14,9 x 13,8 x 16,1	0,99
19	9,1	F	18,3	Crossbreed	53,5	51,4	6,4 x 6,7 x 7,6	0,41
20	6,9	M	32,9	Crossbreed	133,9	89,7	7,5 x 9,1 x 8	0,45
21	9,2	F	36,0	Labrador Retriever	127,7	85,0	4,9 x 5,2 x 6,2	0,29
22	7,4	M	31,0	Golden Retriever	108,5	84,3	16,5 x 19,7 x 12,4	0,76
23	8,2	F	31,7	Belgian Shepherd Malinois	32,5	-50,8	18,4 x 17,5 x 14,9	1,02
24	10,0	M	21,5	English Staffordshire Terrier	18,1	59,6	11,5 x 12,2 x 10,5	0,64
25	7,0	M	10,4	French Bulldog	417,3	N/A	11,5 x 11,1 x 9,5	0,63
26	2,7	F	10, 7	King Charles Spaniel	100	96,0	2 x 6,2 x 4,5	0,29
27	10,7	M	18,5	Border Collie	55	61,8	11,1 x 9,8 x 10,1	0,66
28	8,4	F	33,2	Boxer	126,55	39,8	10,4 x 12 x 8,1	0,49
29	10,2	M	33,4	Boxer	14,5	84,8	5,8 x 4 x 7	0,27
30	7,1	F	44,9	American Bulldog	45,5	31,4	13,7 x 15,8 x 14,8	1,38
31	9,2	F	37,6	Rhodesian Ridgeback	14,8	47,3	13,7 x 13,1 x 13,4	0,78
32	9,7	M	24,8	Crossbreed	N/A	N/A	18,9 x 25,6 x 21,6	1,15
33	7,6	F	22,3	Labrador Retriever	N/A	N/A	14,7 x 9,6 x 9,1	0,93
34	9,9	F	29,1	Labrador Retriever	104,5	4,3	11,4 x 14,5 x 13,2	0,71
35	9,6	M	4,8	Maltese	100,7	97,5	3,4 x 3,8 x 3,2	0,24
36	8,1	F	12,6	Crossbreed	20,5	73,7	19,3 x 15,3 x 15,5	1,4
37	8,9	M	7,4	Australian Terrier	13,85	79,1	4,6 x 5,8 x 5,7	0,3
38	9,3	F	40,0	Boxer	16,5	52,7	11,7 x 12,4 x 13,9	0,7
39	11,0	M	10,0	Shih Tzu	N/A	N/A	10,6 x 15 x 12,9	0,67
40	5,6	M	21,0	Beagle	19,6	93,9	4,2 x 5 x 6	0,26
41	5,9	M	15,8	King Charles Spaniel	383,15	95,1	6,1 x 6,8 x 6,6	0,29
42	10,4	F	19,7	Beagle	361,45	47,0	7,7 x 7,4 x 9,8	0,48
43	8,4	F	6,4	Maltese	57,35	95,8	3,6 x 3,8 x 2,2	0,25
44	10,7	M	25,5	Crossbreed	126	31,0	17,4 x 17,6 x 21,2	0,74

P/B ratio: pituitary-height/brain-area ratio; N/A: not available; C/C12: average of UCCRs of day 1 and 2; % suppression: % suppression of UCCR by dexamethasone; F: female; M: male.

Table 2. Clinical information of the healthy dogs

No.	Age	Sex	Weight (kg)	Breed
45	8m	M	31	Bouvier des Flandres
46	3y8m	F	N/A	Labrador Retriever
47	2y9m	M	N/A	Labrador Retriever
48	3y1m	F	N/A	Labrador Retriever
49	2y9m	F	N/A	Labrador Retriever
50	2y9m	M	N/A	Labrador Retriever

F: female; M: male; N/A: not available; y: year; m:month

Immunohistochemistry

The tissue samples were sent for histopathology (HE-staining) and immunocytochemistry to confirm the diagnosis of PDH as described before. All samples have already been stained with immunohistochemistry for Sox2 and Pax7, during a previous research project.[20]

Expression Analysis for Pax7 and Sox2

The expression of the markers Sox2 and Pax7 was analyzed by calculating the labeling index, which means the number of positive cells/1000 cells.[21] All stained slides have been photographed, and these photos were analyzed using the cell counter option of the Image J software(Last update:2004, National Institutes of Health, Maryland, U.S.A). With this program it´s possible to count the different types of cells in a slide. The cells that were positive for either Sox2 or Pax7 stained brown, the remaining cells stained blue. In each slide, the cells in the left upper corner on 75% zoom were counted. Mostly, not enough cells were in the left corner, so the right upper corner was used to count 1000 cells in total. Per sample a 1000 cells were counted and the number of positive cells/1000 cells (labelling index) was calculated.

Statistical analysis

For statistical analysis, the SPSS software (Version 22, Released: 2013, IBM, New York, U.S.A) is used. For the comparison of the expression of Pax7 and Sox2 between different groups, the Mann- Whitney U test was used. The expression of Pax7 and Sox2 were compared between the group recurrence and the group no recurrence. Also, for Pax7 and Sox2, between the group remission and no remission, non-enlarged and enlarged, anterior lobe and intermediate lobe, not malignant and malignant, and between the group healthy and the group patients.
Mann- Whitney U test is a non-parametric test, which in two different independent groups can be compared.
For testing correlation between different clinical parameters and expression of Pax7 and Sox2 the Spearman correlation is used. The Spearman correlation was investigated between the expression of Pax7 and Sox2 and the disease interval, and for the expression of Pax7 and Sox2 and the survival time, and for the expression between Pax7 and Sox2. The significance is set at P<0,05.
The survival analysis is also tested. The survival analysis is tested for patients with

negative Pax7 expression and for patients with positive Pax7 expression. The results of the groups are compared with each other. For Sox2, the patients with low (<16,5) Sox2 expression and the patients with high (≥16,5)Sox2 expression were tested and the results are compared with each other.

Results

A population of 50 dogs includes, this were 29 males and 21 females, 3 of the female dogs were healthy and 3 of the male dogs were healthy. The median of body weight in the patients was 22,17 kg, (range 4,8-48,2). The median age was 25,1 year, (range 0,8-11,9). The median P/B ratio was 0,62, (range 0,2-1,25).

Expression of Pax7 and Sox

The expression of Pax7 and Sox2 was very different among samples. Some slides showed high expression and others were negative. Also, expression of Pax7 and Sox2 differed greatly (Figure 3). Median labeling index for Pax7 was 8,0%, (range 0-90,8). Median labeling index for Sox2 was 16,8%, (range 0-63,4).

Figure 3. Expression of Pax7 and Sox2 in the patients

The table shows the number of slides in different categories of %positive cells for Pax7 and Sox2.

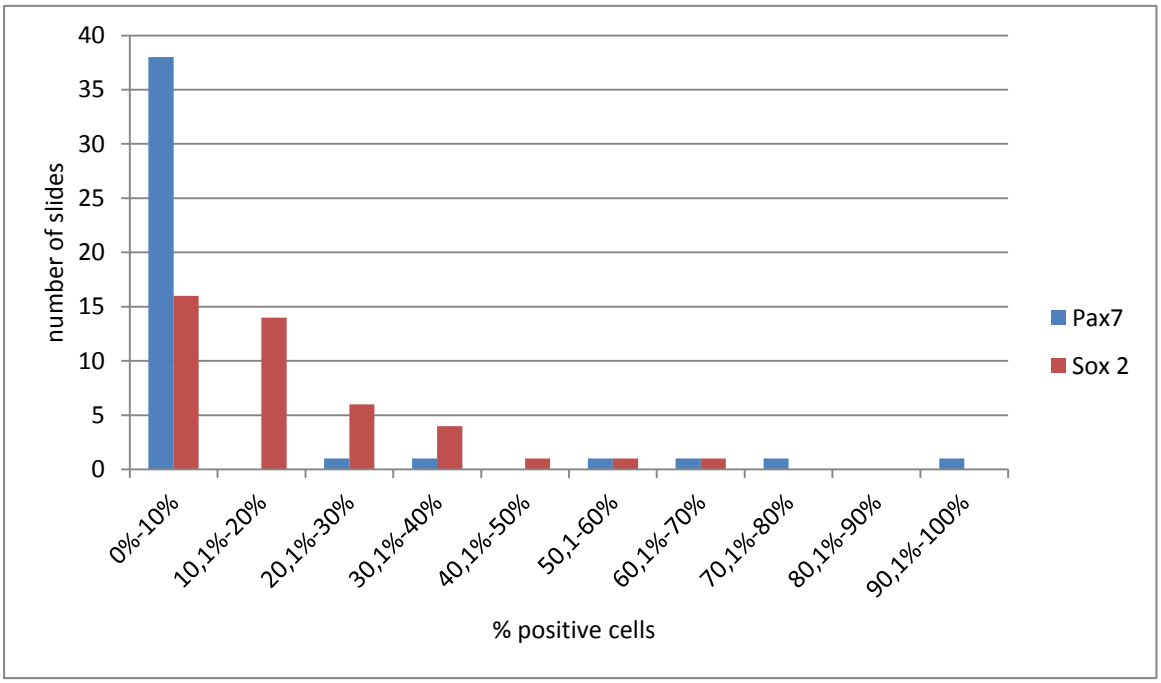


Table 3 shows the follow up of all dogs. Also, the labeling indices of Pax7 and Sox2 in the patients and the healthy dogs and tumor characteristics such as malignance and the location of the tumor in the patients.

Table 3. Follow up data of the patients included

No.	follow up (months)	Dfi (months)	Recurrence	Remission	NE/E	tumor characteristics	%p ositive Pax7 cells	% positive Sox2 cells
1	17,9	14,4	no	yes	NE	AL	0,0	5,9
2	23,8	23,8	no	yes	NE	AL	3,2	5,7
3	50,8	50,8	no	yes	NE	AL	0,0	23,0
4	8,7	8,7	no	yes	NE	AL	0,0	30,0
5	126,9	126,9	no	yes	NE	AL	0,0	18,8
yes 6	2,6	1,5	yes	yes	E	AL	0,0	0,0
7	43,9	30,0	no	yes	E	AL/M	0,0	0,0
8	23,8	23,8	no	yes	E	AL	0,0	39,6
9	27,1	18,9	yes	yes	E	AL/M	0,0	13,7
10	66,5	49,8	yes	yes	E	IL	35,0	18,7
11	15,3	15,3	no	yes	E	AL	0,0	0,0
12	27,4	27,4	no	yes	E	AL/M	2,0	30,7
13	20,6	8,4	yes	yes	E	AL	0,0	10,1
14	0,0	N/A	-	-	E	AL	0,0	0,0
15	28,6	28,6	no	yes	E	AL	66,0	63,4
16	84,4	84,4	no	yes	E	IL	0,0	0,0
17	61,7	40,1	yes	yes	E	AL	8,4	24,0
18	18,8	4,4	yes	no	E	AL	0,0	4,8
19	5,6	5,6	no	yes	E	IL	0,0	19,2
20	76,2	72,1	no	yes	E	IL	2,5	8,8
21	51,6	51,6	no	yes	NE	AL	1,4	17,5
22	5,8	N/A	-	no	E	IL	0,0	0,0
23	11,3	4,4	yes	no	E	AL	6,8	9,7
24	19,3	19,3	no	yes	E	AL	0,0	14,5
25	6,0	5,9	yes	no	E	AL	3,3	17,4
26	0,0	N/A	-	-	NE	AL	0,0	21,3
27	55,6	43,7	yes	Yes	E	AL	0,0	9,0
28	14,7	14,7	yes	Yes	E	AL/M	0,0	28,2
29	14,3	14,3	no	Yes	NE	AL	0,0	15,9
30	5,5	N/A	-	-	E	AL	0,0	1,7
31	27,9	27,9	No	yes	E	AL	90,8	38,4
32	0,1	0,1	-	-	E	AL	76,3	0,0
33	29,3	29,3	no	yes	E	AL	23,3	20,5
34	26,2	18,2	yes	yes	E	AL	0,0	13,9
35	18,2	18,2	no	yes	NE	AL	0,0	52,7
36	0,1	0,1	-	-	E	AL	0,9	0,0
37	8,7	7,7	no	yes	NE	AL	0,3	19,8
38	5,9	5,9	yes	yes	E	AL/M	0,0	14,5
39	18,4	18,4	no	yes	E	AL	0,0	19,4
40	27,6	27,6	no	yes	NE	AL	0,0	0,0
41	23,6	23,6	no	yes	NE	AL	0,0	N/A
42	15,9	15,9	no	yes	E	IL	50,7	49,5
43	25,0	25,0	no	yes	NE	AL	0,0	14,9
44	0,4	N/A	-	-	E	IL	0,0	30,7

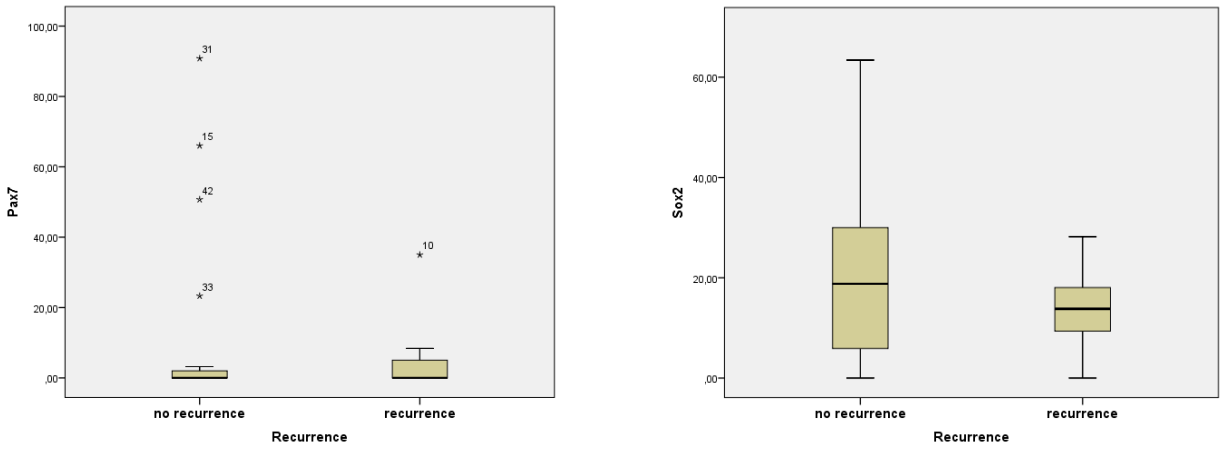
45	-	-	-	-	-	-	18,7	25,4
46	-	-	-	-	-	-	0,0	12,2
47	-	-	-	-	-	-	0,0	0,0
48	-	-	-	-	-	-	0,0	21,5
49	-	-	-	-	-	-	15,2	32,4
50	-	-	-	-	-	-	0,0	25,2

DFI: disease free interval; NE: non-enlarged adenomas; E: enlarged adenomas; AL: anterior lobe; IL; intermediate lobe; M: malignant tumor; N/A: not available

Recurrence vs. No recurrence

First, the expression of Pax7 and Sox2 is compared between the group of patients that had recurrence and the group of patients that had no recurrence. No significant difference was found for Pax7 (P=0.970) or Sox2 (P=0.255). Boxplots are made for Pax7 and for Sox2 (Figure 4).

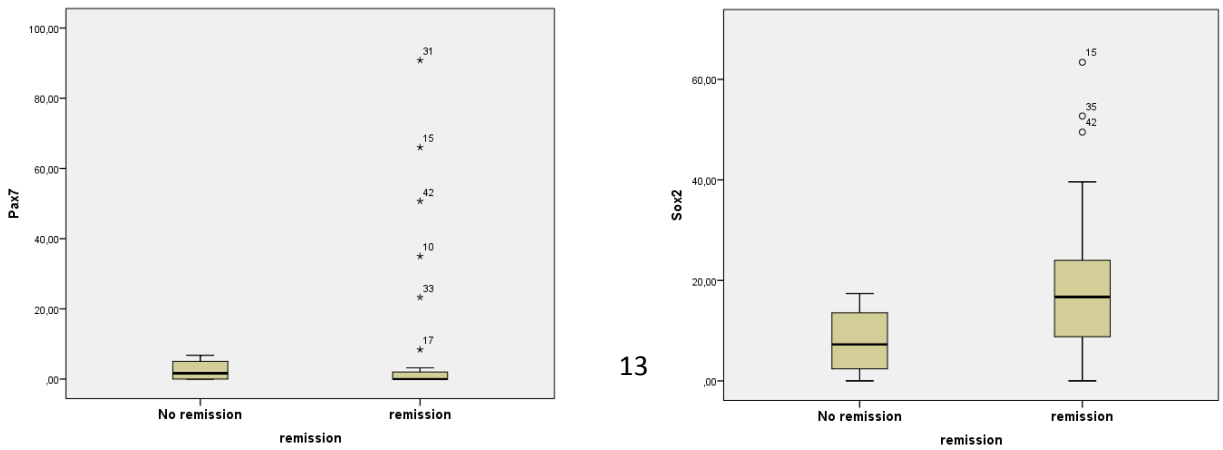
Figure 4. Boxplot of Pax7 and Sox2 in the groups recurrence and no recurrence



Remission vs. no remission

Next, the groups of patients that had remission and the group of patients that had no remission are compared with the expression of Pax7 and Sox2. Also, no significant difference was found for Pax7 (P=0.536) and for Sox2 (P=0.127). Figure 5 shows this by using a boxplot.

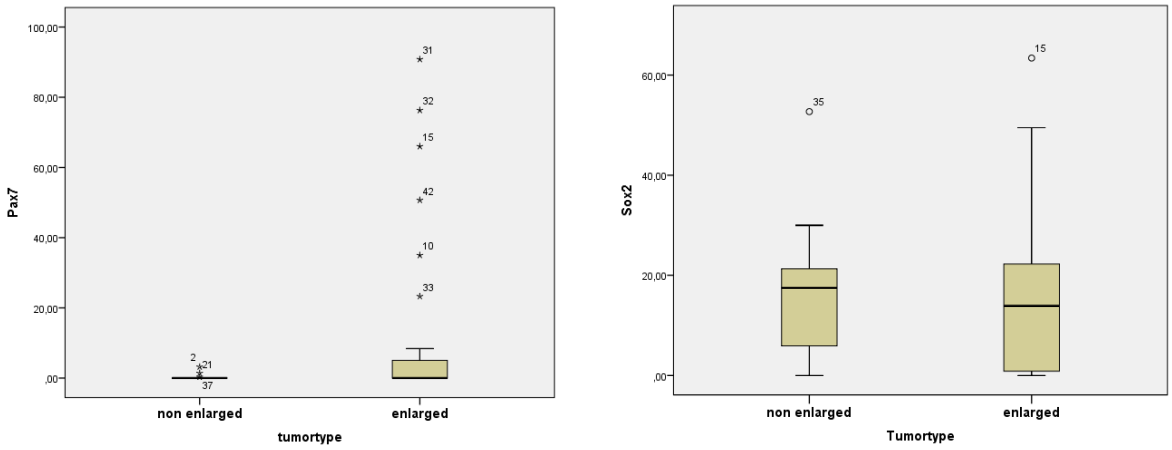
Figure 5. Boxplot of Pax7 and Sox2 in the groups remission and no remission



Enlarged vs. Non- Enlarged

The group of patients with an enlarged tumor and the group of patients with a non-enlarged tumor is compared with the expression of Pax7 and Sox2.
No significant difference was found for Pax7 (P=0.166) and for Sox2 (P=0.526).

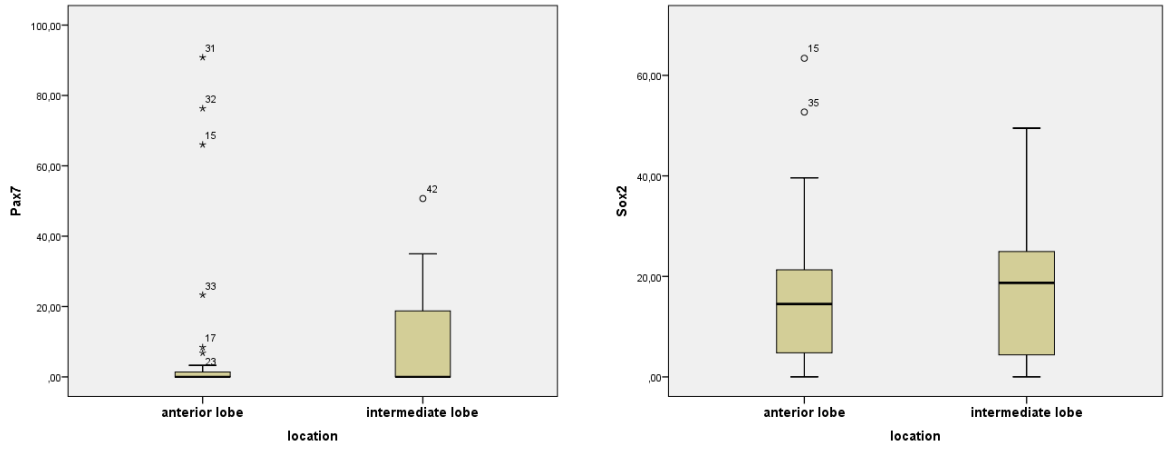
Figure 6. Boxplot of Pax7 and Sox2 in the groups enlarged and non- enlarged



Location of tumor

The expression of Pax7 and Sox2 can also be compared different locations of the tumor. The group of patients with the tumor in the anterior lobe and the group of patients with a tumor in the intermediate lobe is compared with the expression of Pax7 and Sox2. No significant difference was found for Pax7 (P=0.506) and for Sox2 (P=0.872)(Figure 7).

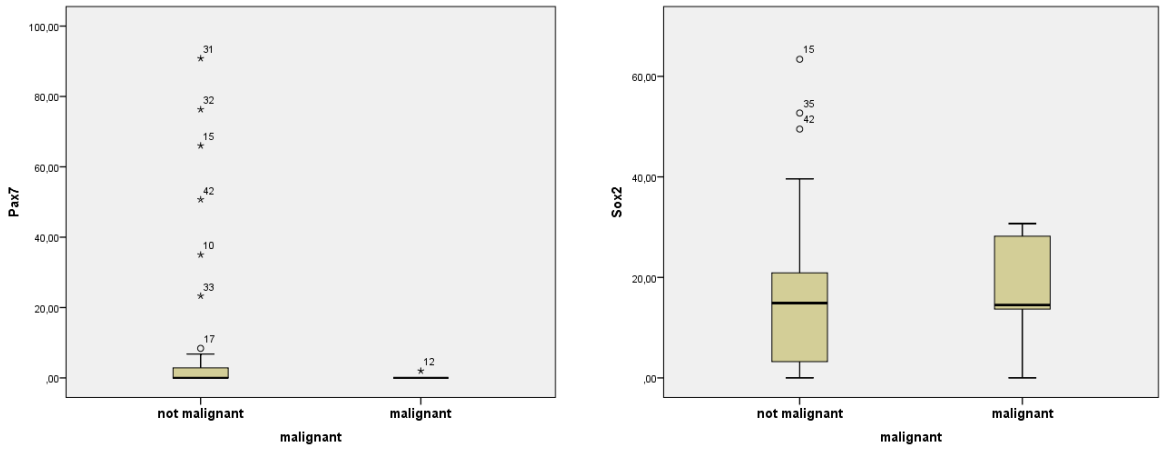
Figure 7. Boxplot of Pax7 and Sox2 in the groups anterior lobe and intermediate lobe



Malignancy

Some patients had tumors with malignancy characteristics, this group is and the group of patients with no malignant tumors is compared with the expression of Pax7 and Sox2. Also this time, no significant difference was found for Pax7 (P=0.393) and for Sox2 (P=0.766).

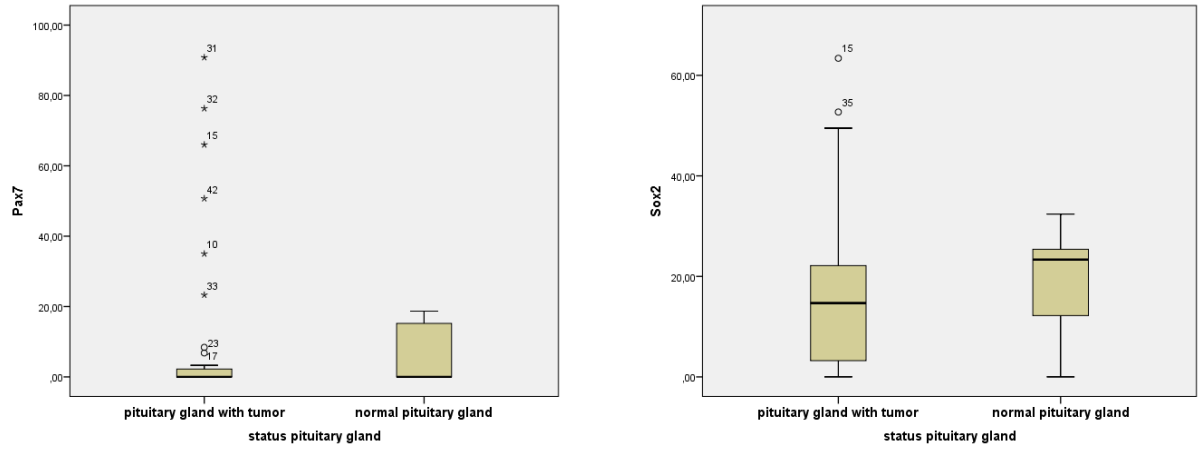
Figure 8. Boxplot of Pax7 and Sox2 in the groups not malignant and malignant



Normal pituitary gland vs. Pituitary gland adenoma

The group of dogs with a pituitary gland adenoma and the group of dogs with a normal pituitary gland is compared with the expression of Pax7 and Sox2. No significant difference was found for Pax7 (P=0.944) and for Sox2 (P=0.322).

Figure 9. Boxplot of Pax7 and Sox2 in the groups dogs with pituitary gland adenoma and dogs with a normal pituitary gland



Disease free interval

The correlation between the disease free interval and the expression of Pax7 and Sox2 is tested. No significant correlation was found for Pax7 and Sox2. The correlation coefficient (r_s) for Pax7 is 0.209 (P=0.173) and for Sox2 it's 0.207 (P=0.177).
A scatterplot is made to see if there is a relationship between the two parameters(Figure 10).

Figure 10a. Scatterplot of correlation between labeling index Pax7 cells and disease free interval

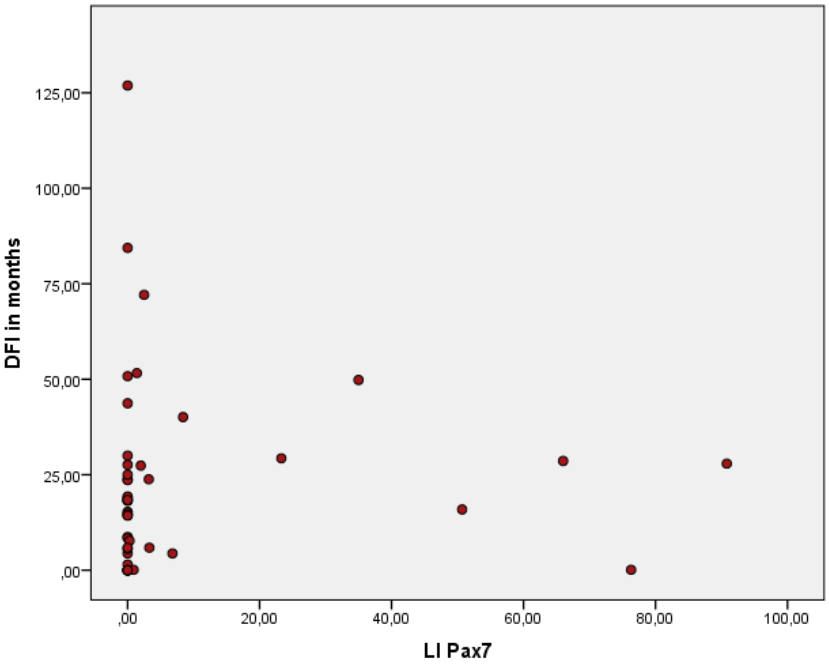
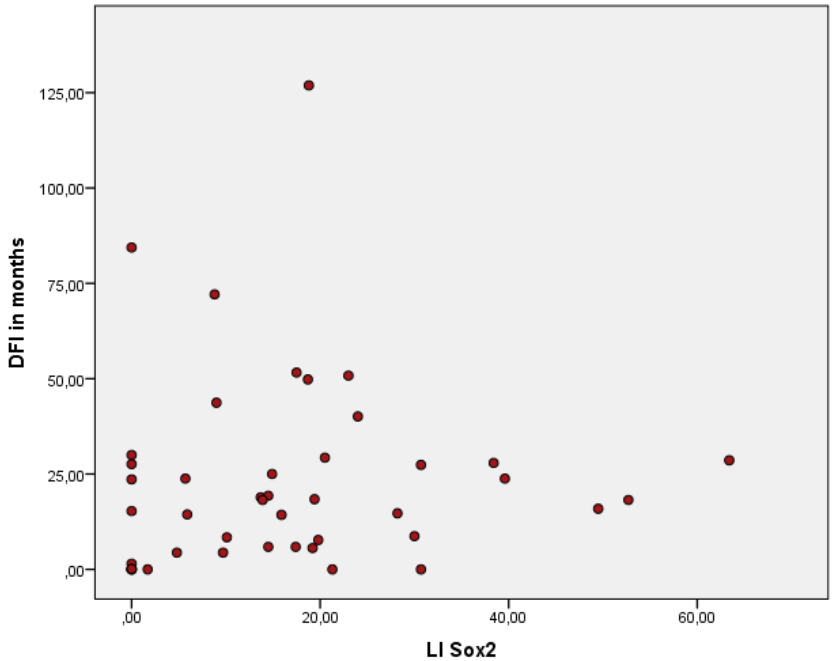


Figure 10b. Scatterplot of correlation between labeling index Sox2 cells and disease free interval



Survival time

The correlation between the survival time and the expression of Pax7 and Sox2 is also tested. Only the death patients are included.
No significant correlation between the expression of Pax7(r_s=0.345, P=0.062) and the survival time was found. A significant positive correlation for Sox2(r_s=0.419, P=0.021) was found. Figure 11 shows the scatterplot of Pax7 and Sox2 and the relation between the length of the survival time.

Figure 11a. Scatterplot of correlation between labeling index Pax7 cells and survival time

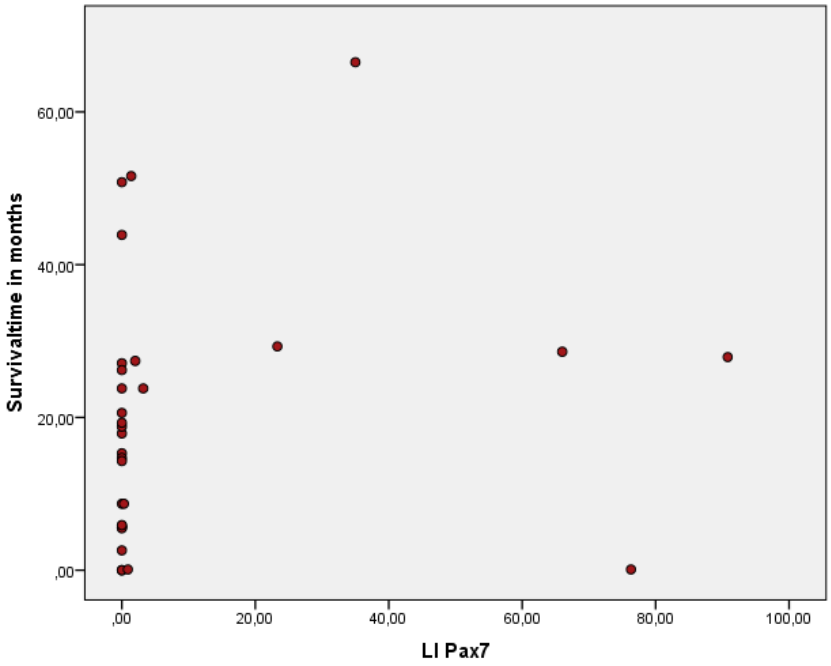
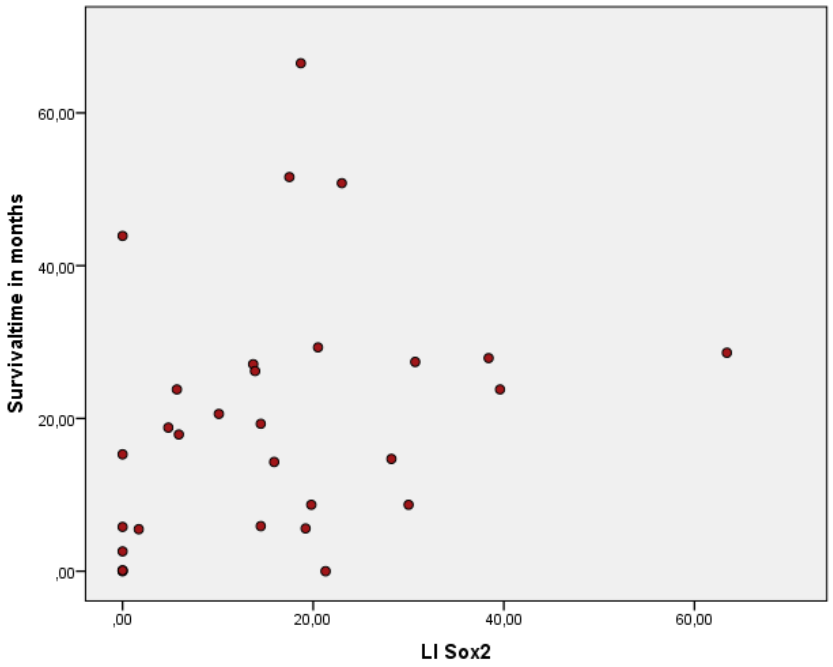


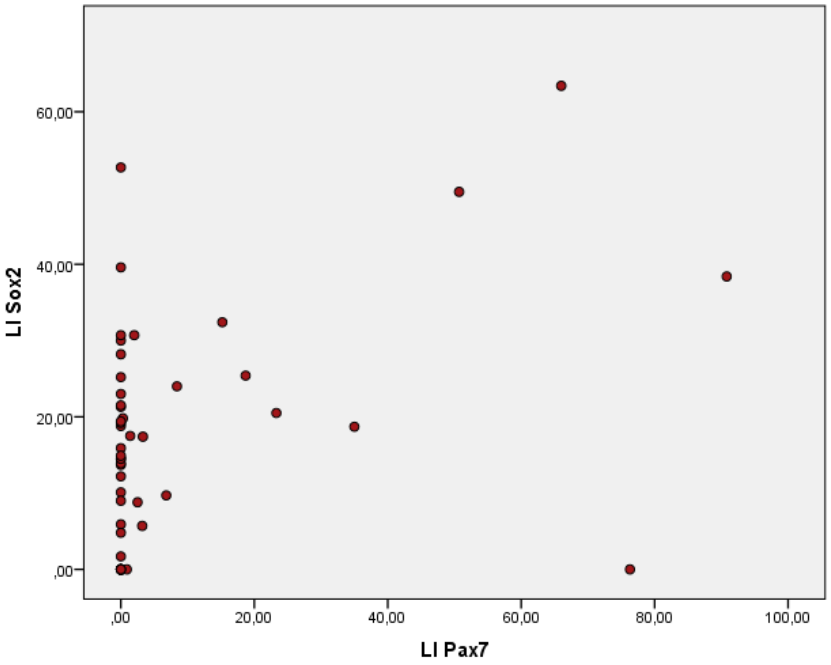
Figure 11b. Scatterplot of correlation between labeling index Sox2 cells and survival time



Relation between expression Pax7 and Sox2

Also, the expression of Pax7 and the expression of Sox2 are compared with each other. A significant positive correlation is found(r_s =0.300, P=0.034). A scatterplot is made to show the correlation between Pax7 and Sox2(Figure 12).

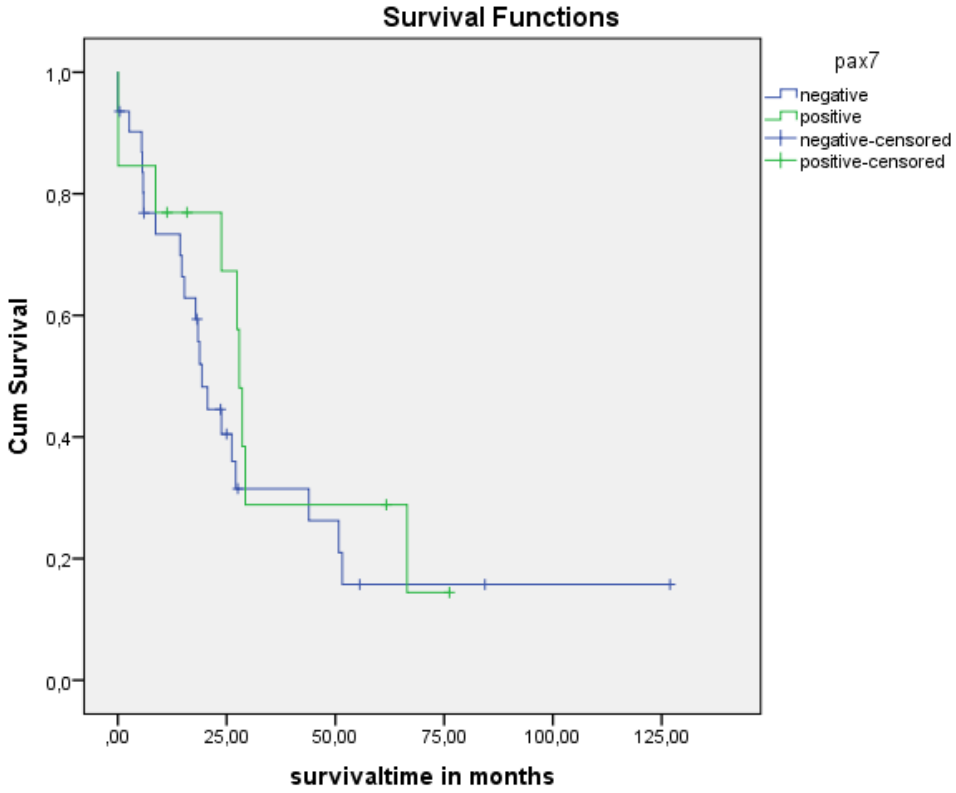
Figure 12. Scatterplot of correlation between labeling index Pax7 cells and labeling index Sox2 cells

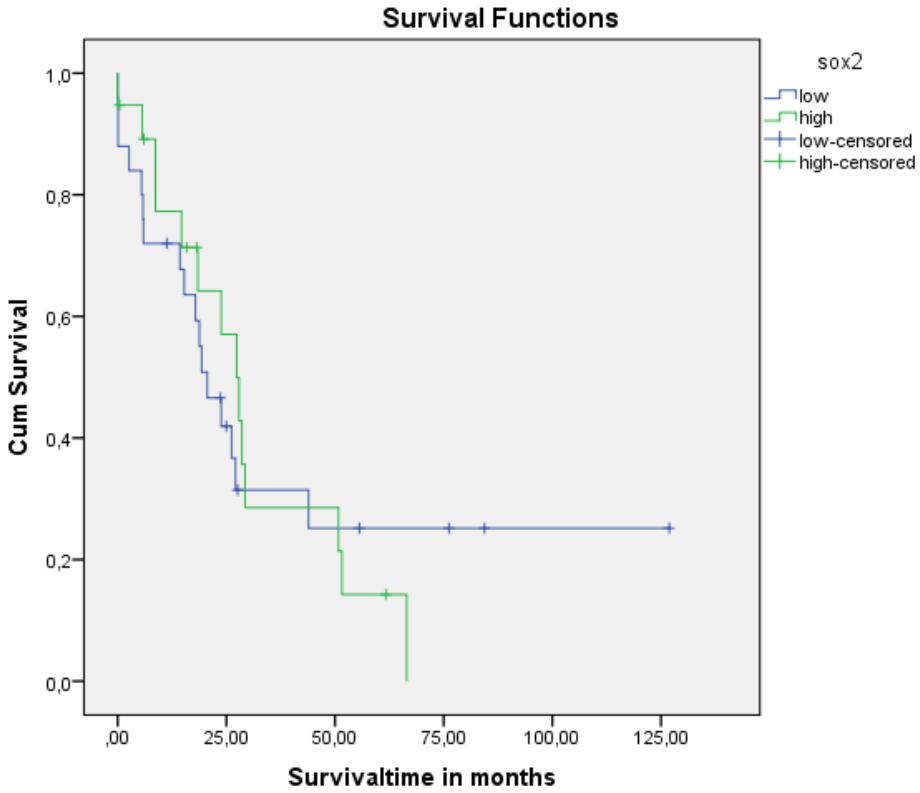


Survival analysis

A survival analysis is made for Pax7 and for Sox2. For Pax7, a difference is made for Pax7- and for Pax7+. For Sox2, the difference is made for a high expression(≥ 16,5) of Sox2 and a low expression(<16,5)of Sox2. The average survival time for Pax7- patients was 19,3 months and for Pax7+ patients 27,9 months. No significant difference was found between Pax7+ en Pax7- (P=0.390). Thus, nothing can be concluded from the graph(Figure 13).
The average survival time for patients with low expression was 20,6 months and for the patients with a high expression of Sox2 27,4 months. However, no significant difference in survival time for high or low expression was found(P=0.983).

Figure 13. Survival analysis for Pax7 and for Sox2





Discussion

The aim of this research project was to study if the transcription markers Pax7 and Sox2 had a correlation with clinical parameters such as, tumor type, the size of the pituitary gland, disease free interval of the dog, survival time of the dog and recurrence, remission of the tumor after surgery. This can give more knowledge about the usefulness of these markers in giving prognosis and prediction of outcome in dogs with a pituitary adenoma.

The result for Pax7 was that there was no significant difference between the groups that were compared.
When the groups were compared for Sox2 there was also no significant difference between those groups. Notable is, that when the groups normal pituitary gland and pituitary gland adenoma were compared there was also no difference.
The outcome is remarkable because this is in contrast with our hypothesis that when there's a high expression of Pax7 and Sox2 in pituitary gland adenomas, the prognosis for the dog is less favorable.

When correlation was used to compare disease free interval with the height of the expression of Pax7 and Sox2, there was also no correlation between the parameters.
The clinical parameter survival time shows a positive relation between the expression for Sox2 but not for the expression of Pax7. The positive relation between survival time and the expression of Sox2 is remarkable and also in contrast with the hypothesis. The clinical relevance of this result is still unclear.
A significant correlation is found between Sox2 and Pax7.

The survival analysis for Pax7 shows no significant difference between the groups positive Pax7 and negative Pax7. For Sox2, also no significant difference was found between the groups low expression and high expression of Sox2. This is remarkable because this is in contrast with the hypothesis that patients with a higher expression of Pax7 and Sox2 had a less favorable prognosis. Now, the outcome shows no difference.
A possible explanation for the outcome of Sox2 is that there was trauma in the pituitary gland and Sox2 may play a part in the regenerating after trauma according to a study of Alatzoglou et al.[16] So this can affected the rate of expression of Sox2 and our outcome.

The results that are found are dependent on the tissue samples that were collected during surgery. Also they are dependent on the previous research, during this research the samples were stained with immunohistochemistry for Sox2 and Pax7. It could be possible that, accidentally, not the right tissue with the most positive cells was included. So this had an effect on the results in this research.
During the cell counting there could be made a mistake in cell counting. There were cells that were not clearly brown or blue so they could be classified in the wrong group. Some parts in the samples were damaged so this had possible also effect on the results because maybe there was a higher percentage positive cells when there wasn't an artefact.
Also were some cells lighter brown or dark brown maybe this is a difference in the rate of expression but it should be possible that the lighter brown cells are another type of cell.
In this study 1000 cells were counted in each sample at the same place, it would be more reliable if all cells in the tissue sample were counted. To perform this, it would take too

much time for this research.

In the study of Budry et al[14], it was investigated that 30% of the canine pituitary gland adenomas express Pax7. When this is compared with the results from this study, the outcome is circa the same. 15 canine pituitary gland adenomas were positive tested for Pax7 and this is 34% of the canine pituitary gland adenomas.

There were positive Pax7 cells present in the anterior lobe and the intermediate lobe, in comparison with the study of Hosoyama et al[13], where positive Pax7 cells were localized in the intermediate lobe and in the between the anterior lobe and the pars intermedia, the comparison is that we also have Pax7 expressing cells in the intermediate lobe.

Conclusion

The conclusion in general is that there is no evidence found for the hypothesis that the prognosis is less favorable when there is a high expression of Pax7 and Sox2.
All clinical parameters that were compared with the expression of Pax7 and Sox2 shows no significant difference. This means that it has no effect on the prognosis of the dogs.
An unexpected finding was that there was a significant positive relation between the expression of Sox2 and the survival time.
More research is necessary to investigate the expression of Pax7 and Sox2 in canine pituitary gland adenomas and also for their role in pituitary tumorigenesis because this is still not clear.

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