Research minor
Master 'Companion Animals'
Faculty of Veterinary Medicine
Utrecht University

March - October 2014

The search for stem cells in canine pituitary tumors

1. Optimization of the FACS technique and isolation of a side population of canine healthy pituitary and pituitary adenoma

2. RNA quality after FACS analysis of canine healthy pituitary and pituitary adenoma cells

3. The role of PC2 and Pax7 in pituitary adenomas

Pouwer, M.G.

1. Contents

2. Summary

Pituitary-dependent hypercortisolism (PDH) or Cushing's disease is a common endocrinopathy is dogs, caused by an ACTH-producing pituitary tumor and has a reported incidence of one to two cases per 1000 dogs per year. Clinical signs of PDH include polyuria/polydipsia, muscular atrophy, weight gain, abdominal enlargement, thin coat, alopecia and lethargy. Treatment consists of eliminating the glucocorticoid excess with a medical treatment with Trilostane (Vetoryl®), or by eliminating the pituitary lesion via transsphenoidal hypophysectomy. The disadvantage of medical treatment is that the tumor remains in place and can cause neurological problems with expansion. With the removal of the tumor both endocrine and neurological problems are eliminated, but dogs will need lifelong hormone supplementation therapy. Although postoperative remission rate is high, long term recurrence of the disease is common (around 23%).

It is hypothesized that Cancer Stem Cells (CSC) are a subpopulation of cells that are responsible for growth, maintenance and progression of tumors. Hypophysectomy and the availability of pituitary tumor tissue enable the isolation and characterization of these stem cells. In this way, new prognostic markers can be identified and ultimately, they could be used as new therapeutic targets, making therapy less invasive for the patient and more efficient against the disease. So far, no specific markers are identified to isolate stem or progenitor cells in the canine pituitary gland.

By means of a technique called Fluorescence Activated Cell Sorting (FACS), a small cell fraction (side population, SP) can be separated from the majority of cells (main population, MP). SP cells have been isolated from different tissues, and many times, the SP appeared to be enriched in stem cells. Previously, the SP from canine healthy pituitaries was successfully isolated.

In the present study, the cell digestion protocol and FACS technique was optimized for canine healthy pituitary and pituitary adenomas. Two different FACS instruments were used and several methods were tested to collect the cells after sorting, in order to obtain high quality RNA. There were high variations in percentage life cells and total life cell count among the samples. These variations may have influenced the SP appearance and with that, the proportion of sorted SP cells. When sorting bone marrow cells, RIN values of >6.5 were obtained from samples collected in RLT-buffer + β-mercaptoethanol (β -ME), empty tubes or in 1 ml RNA *Later®*, whereas RIN values were much lower in sorted pituitary gland samples. There was no significant difference in obtained RNA yields and quality between pituitary samples sorted with different the FACS machines or collected by different collection methods. However, fresh samples sorted on the BD Influx showed a more stable expression of the reference genes *tbp, hmbs* and *ywhaz*, compared with frozen samples sorted on the FACSAria II (SORP).

Next to the FACS study, the role of Pax7 and PC2 in canine pituitary adenomas was investigated. In mice, Pax7 expression is restricted to the melanotroph cells of the intermediate lobe. In canine, Pax7 expression was found in one healthy pituitary, and not in all intermediate lobe adenomas. Therefore, expression of the melanotrophe protein PC2 was investigated, but no expression was found in healthy pituitaries or pituitary adenomas. Besides, decreased gene expression of Pax7, Drd2, PC2, Sox2 and Thbs2 was found in canine pituitary adenomas, compared with healthy pituitaries. These results cannot support in an explanation for the Pax7 expression found in canine

samples, but evoked new research questions about variations in expression of the investigated genes between the different species.

3. General introduction

3.1 The pituitary gland

The pituitary gland is a small endocrine gland located in the pituitary fossa, a depression in the sphenoid bone. The pituitary gland consist of three functional units, the anterior lobe (AL) (pars infundibularis and pars distalis of the adenohypophysis), the intermediate lobe (IL) (pars intermedia) and posterior lobe(PL) (neurohypophysis) [1]. The adenohypophysis contains of six different cell types, each of them producing and secreting different hormones. The somatotropes secrete growth hormone (GH), the corticotropes secrete adrenocorticotrophic hormone (ACTH), the lactotropes produce prolactin (PRL), the thyrotropes produce thyroid-stimulating hormone (TSH) and thyroid hormone (TH) and last, the gonadotropes secrete luteinizing hormone (LH) and follicle-stimulating hormone (FSH). The intermediate lobe consists of α-melanoctye stimulating hormone (α-MSH) producing melanotropes, and in the posterior lobe arginine vasopressin (AVP) and oxytocin are secreted [2].

The embryological development of the pituitary gland is similar for all mammals. During gastrulation three layers are formed, endoderm, mesoderm and ectoderm. The ectoderm gives rise to the central nervous system (CNS) and peripheral nervous system. The neural plate, a thickening of the ectoderm, folds and forms the neural tube. The anterior two-third of the neural tube develops into the brain. Primary brain vesicles are formed, the diencephalon, telencephalon, mesencephalon, metencephalon and myelencephalon. The neurohypophysis is formed by a ventral down growth of the diencephalon, the infundibulum [3]. The adenohypophysis is formed by the formation of the Rathke's pounch, a fingerlike invagination of the roof of the oral cavity, towards the diencephalon [4]. The infundibulum and Rathke's pounch (RP) together form the definite pituitary gland. The hypothalamus originates from the lateral walls of the diencephalon, which form a groove called hypothalamic sulcus. The surface of the hypothalamus gives rise to the tuber cinereum, which descents and forms the infundibulum or pars proximalis neurohypophysis [5]. The infundibulum connects the posterior lobe of the pituitary gland with the hypothalamus [4].

The hypothalamus-pituitary-adrenal (HPA) axis plays a major role in the body's response to stress. As a response to stress, the hypothalamus secretes corticotropin releasing hormone (CRH). CRH is carried by the hypothalamic axons and secreted in the pituitary portal blood system [2]. Through two different pathways, CRH causes an influx of extracellular calcium into the pituitary cells which is a strong stimulator for ACTH synthesis and secretion [5]. ACTH is derived from the precursor proopiomelanocortin (POMC), which is synthesized in both the corticotrophic cells in the AL as well in cells of the pars intermedia. Thus, a ACTH-secreting adenoma causing PDH may originate in the AL, PI or occur in both lobes. In about 20-25% of the cases there is an adenoma in the PI [2].

3.2 Clinical signs and diagnosis

Clinical signs of PDH are related to the excess of ACTH, the space-occupying effects of the mass, or both. ACTH stimulates the adrenals to produce cortisol, causing hypercortisolism, and clinical signs include polyuria/polydipsia, muscular atrophy, weight gain, abdominal enlargement, thin coat, alopecia and lethargy [2]. With the expansion of the tumor, mass-related clinical signs may occur

and include periods of obtundation, decreased appetite, seizures, anisocoria, aggression, circling and head tilt [6].

Diagnosis of PDH is based on measurement of the urinary corticoid:creatinine ratio (UCCR). The UCCR is measured in two morning urine samples and elevated levels of the UCCR indicate for hypercortisolism. To distinguish between ACTH dependent – or independent hypercortisolism, an oral high-dose dexamethasone suppression test is needed. After collection of the 2nd morning urine sample the dogs receive 3 doses of 0,1 mg dexamethasone/kg PO at 8- hours intervals. A third morning urine sample is taken and when the UCCR is less than 50% of the mean of the first two samples, PDH is diagnosed [2]. To locate the tumor, imaging can be used, such as computed tomography (CT) or magnetic resonance imaging (MRI). CT has a preference over MRI, because bone structures are clearly visible which can be used as landmarks during hypophysectomy. The pituitary gland lies outside the blood-brain barrier so radiographic contrast medium can be used to visualize the tumor. A healthy pituitary gland is 6-10 mm in length, 5-9 mm in width, and 4-6 mm in height. By measuring the height of the pituitary gland and the area of the brain the pituitary/brain (P/B) ratio can be calculated. The P/B ratio can be used to distinguish between enlarged and non-enlarged tumors [7]. Pituitary imaging may not be used for diagnosis, since a normal pituitary appearance does not exclude the presence of microadenomas.

3.3 Treatment

Treatment of PDH consists of eliminating the glucocorticoid excess by a medical treatment with o,p'-PPP (Mitotane®) or Trilostane (Vetoryl®), or by eliminating the pituitary lesion by radiotherapy or hypophysectomy. For years, Mitotane® has been the medical treatment of choice, but in 2002 Trilostane has been reported to be safe and effective and is currently widely used in the treatment of hypercortisolism [8]. The chemotherapeuticum Mitotane® binds covalently to adrenal proteins before being converted into a radioactive metabolite, which causes destruction of the adrenal cortices. Dependent on the dose and frequency, the destruction is selective or non-selective. With a selective therapy, only the two inner zones of the adrenal cortices are destructed, sparing the mineral-corticoid-producing zona glomerularis. After an induction period, a life-long maintenance dose of Mitotane® is needed [9]. If a non-selective therapy is used, all zones are destructed and a life-long substitution therapy with cortisone and sodium chloride is needed due to induction of hypocortisolism [10]. In table 3.1, the 1-, 2-, and 3-year survival fractions of the different therapy possibilities are listed. A non-selective protocol gives a longer survival fraction compared with the partial destruction of the adrenals. However, 11.6% of the patients died in the induction period of the non-selective treatment protocol due to over dosage [10]. Other disadvantages of Mitotane® are the severe side effects and administration had to be stopped temporally in 29% of the patients [10]. Besides, administration of the drug is not without risk for the patient's owners.

Trilostane is a competitive inhibitor of 3β-hydroxy-steroid dehydrogenase which interrupts the synthesis of the hormone cortisol. First, the optimal dose of Trilostane is adjusted based on clinical signs and results of the ACTH stimulation test. When the optimal dose is achieved, life-long maintenance therapy is needed. Barker *et al* [9] compared the use of Trilostane with a selective protocol with Mitotane® for the treatment of PDH and survival fractions are more or less the same for both protocols (table 3.1). Although this study is the only study published that compares administration of Trilostane with an alternative treatment, other studies have shown Trilostane to

be effective in the treatment of PDH. Less side effects are seen [11,12], cortisol plasma ratio decreases significantly [11] and a 1-year survival fraction of up to 75% is found [8]. Based on these results and the safety of the drugs for both patient and owner, Trilostane is the most common drug used in the treatment of hypercortisolism.

The disadvantage of a medical treatment, are the neurological problems that may occur if the tumor expands. In dogs diagnosed with a pituitary mass, neurological signs are observed in 30% to 58% of the cases [6,13,14]. To decrease tumor size, radiation therapy can be used. In a retrospective study of Kent *et al* [6], dogs that underwent radiation therapy were compared with dogs receiving no radiation therapy. Dogs were treated in a Monday-through-Friday schedule with a total dose of 48 Gy and presence of neurological and endocrine clinical signs was evaluated pre and post treatment. Various treatments, including Mitotane®, were used in the group of dogs that did not receive radiation therapy. A prolonged survival time was found in the group that received radiation therapy (Table 3.1) and 70% of the dogs had transient or complete resolution of neurological signs. However, tumor size was not evaluated after irradiation and only 5 out of 14 dogs had resolution of clinical signs related to hypercortisolism. In a study of Fornet *et al* [14] the effect of radiation on tumor size was investigated. Dogs received radiation therapy and additional medical treatment with Mitotane® or ketaconazole. A decrease of more than 30% in tumor high was found in 11 of the 12 dogs reexamined. In all dogs, clinical signs of hypercortisolism and neurological signs resolved. These studies indicate that radiation therapy is successful to reduce tumor size and associated neurological signs in dogs diagnosed with a pituitary mass, but not to decrease clinical signs of hypercortisolism. Therefore, additional medical treatment with Mitotane® or Trilostane is needed.

Hypophysectomy is the complete removal of the pituitary gland and eliminates both neurological and endocrine signs. The 1-, 2-, and 3-year survival fractions of radiation therapy and hypophysectomy are more or less the same (Table 3.1), with a higher 1-, and 2- year survival fraction after radiation therapy [6] and a higher 3- year survival fraction after hypophysectomy [15]. After hypophysectomy higher survival fractions were found compared with medical treatment, and an overall success of 65%. A disadvantage of surgery is that a lifelong hormone replacement is needed with cortisone acetate and thyroxine. In our clinic, hypophysectomy is the treatment of choice.

Therapy	1-,2-,3-year survival fractions	1-,2-,3-disease free fractions	Recurrence rate	Overall success	Number of dogs in study	reference
Mitotane® (Non-selective)	80% 69% 61%	77% 53% 44%	39%	61%	111	Hertog E. *Vet Rec* 1999:144;12-17
Mitotane® (Selective)	62% 48% -	-	-	-	148 (25 on Mitotane®)	Barker EN. *J Vet Intern Med*. 2005;19:810-15
Trilostane	68% 47% -	-	-	-	148 (123 on Trilostane)	Barker EN. *J Vet Intern Med*. 2005;19:810-15
Radiation	93% 87% 55%	-	-	-	19	Kent MS. *J Vet Intern Med* 2007;21:1027-33
No radiation, various treatments	45% 32% 25%	-	-	-	27	Kent MS. *J Vet Intern Med* 2007;21:1027-33
Hypophysectomy	84% 76% 72%	88% 75% 66%	25%	65%	150	Hanson JM. *J Vet Intern Med*. 2005:19-687-94

Table 3.1: The 1,- 2,- and 3- year survival fractions of the different therapies commonly used for the treatment of PDH.

3.4 Transphenoidal hypophysectomy

Transphenoidal hypophysectomy is performed via a transsphenoidal approach as described previously by Meij *et al* [7,16](figure 3.1). During this procedure, the dog is positioned in sternal recumbency and the position of the burr hole is determined by the following landmarks: the hamular processes and the ridge on the sphenoid bone. In mesocephalic dogs the caudal part of the pituitary gland is located to the end of the hamular processes and the cranial part of the tumor at the point where the surface of the sphenoid bone becomes flat [7]. First, an incision is made in the soft palate, in the midline of the hamular processes. Next, the mucoperiosteum is incised making the presphenoid and basisphenoid bone visible. The location of the burr hole is determined and burring is discontinued before the inner cortical layer. The thin inner cortical layer is opened using a ball-tipped hook and an incision is made in the dura mater making the pituitary protrude through the opening. The pituitary is carefully detached from the pituitary fossa and the fossa is inspected to make sure the adenoma is removed completely. To close the wound and to prevent post-operative bleeding, the pituitary fossa is filled with absorbable gelatin sponge and the burr slot in the sphenoid bone is filled with bone wax. The soft palate is sutured in two separate layers.

Immediately after surgery hormone replacement is started with hydrocortisone, a cortisol analog, and desmopressin, a AVP analog. As soon as the dog starts eating an oral maintenance therapy is started with cortisone acetate and thyroxine [16]. The dose of hydrocortisone is gradually tapered until a maintenance dose is achieved. AVP is produced by the hypothalamus, but secreted by the pituitary gland. Desmopressin is administered for 2 weeks as a drop into the conjunctival sac every 8 hours until AVP secretion is regulated by the hypothalamus. In some dogs AVP secretion is not regulated well and they develop central diabetes insipidus (CDI). Other complications of hypophysectomy are procedure-related mortalities, incomplete hypophysectomy and postoperative

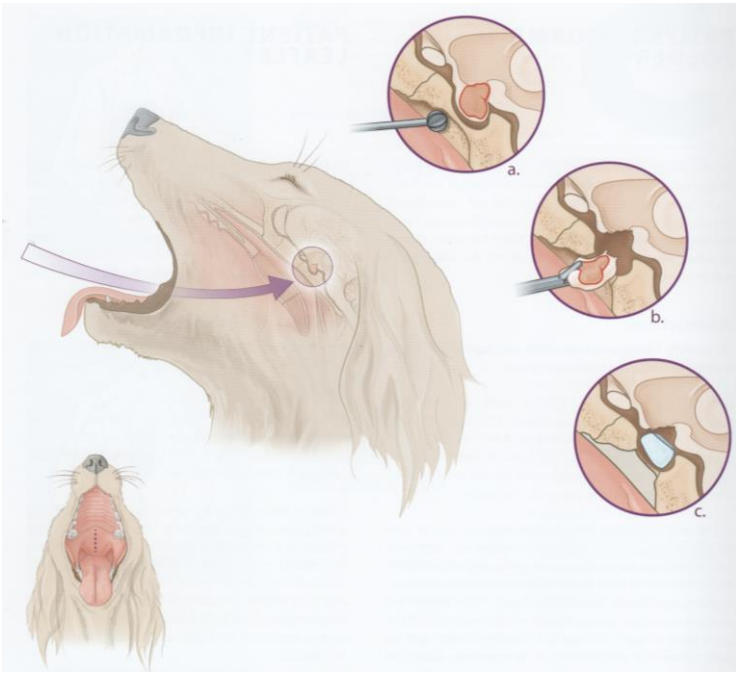


Figure 3.1: Transphenoidal hypophysectomy is performed via a transsphenoidal approach. (A) An incision is made in the soft palate, the mucoperiosteum is incised making the presphenoid and basissphenoid bone visible and burring is discontinued before the inner cortical layer. (B) The thin inner cortical layer is opened using a ball-tipped hook and an incision is made in the dura mater making the pituitary protrude through the opening (C) the pituitary fossa is filled with absorbable gelatin sponge and the burr slot in the sphenoid bone is filled with bone wax. *Remko van Deijk; transsphenoidal hypophysectomy in dogs and cats*

reduction of tear production [15]. In a study of Hanson *et al* [17], prognostic factors for outcome after hypophysectomy were investigated. Date collected between 1993 and 2005 of a total of 181 dogs that underwent hypophysectomy were analyzed and large pituitary size, thick sphenoid bone, high UCCR and high concentration of plasma α-MSH before surgery were associated with an increased risk of disease recurrence. Although a remission rate of respectively 75% [15] and 85.6% [17] was found, long term recurrence is common. Of the dogs that went in remission after hypophysectomy, recurrence is seen in 23% [17]. Therefore, it is important to find prognostic factors which can be used as new therapeutic targets or to predict recurrence.

3.5 The search for proliferation markers

In the past years, several proliferation markers have been investigated in the canine pituitary gland and corticotroph adenoma (Table 3.2). Tpit, a marker specific for POMC-expressing cell lineages, regulates the late differentiation of corticotroph and melanotrophs cells. Tpit expression is found in corticotrophs and melanotrophs in both the normal canine pituitary as well as in corticotroph adenomas. There are no mutations found in the Tpit gene that play a role in the development of pituitary adenomas [18]. Another candidate gene studied is Leukemic Inhibitory Factor (LIF), which inhibits the proliferation of leukemias, but acts like a growth factor in several neoplasms [19]. LIF promotes corticotroph proliferation during embryogenesis as well as in the adult pituitary and induces POMC transcription and ACTH-secretion. Although there is a strong co-expression between LIF and POMC, no significant difference is found between LIF expression in healthy pituitaries and corticotroph adenomas [19].

Another marker of the POMC-lineage investigated is Epidermal Growth Factor Receptor (EGFR). EGFR is a transmembrane protein and binding with the ligand leads to cell proliferation. Although 60% of human pituitary tumors express EGFR [20], the role of EGFR in pituitary tumor pathogenesis is unclear. In an in vitro study of Fukuoka *et al* [21] canine cell cultures derived from 12 different ACTH-producing adenomas where treated with gefitinib, a tyrosin kinase inhibitor (TKI). TKI targets EGFR and blocks the activity of the intracellular ATP-binding site of the tyrosine kinase domain,

suppressing POMC expression. This suppression was demonstrated by dose dependent suppressing of POMC mRNA levels and inhibition of ACTH secretion in the culture medium. Of the tumors responding to gefitinib, EGFR expression was found [21].

In 2010, the expression of the cell cycle inhibitor p27kip1 and of the proliferation markers proliferating cell nuclear antigen (PCNA) and Ki-67, was for the first time investigated in canine pituitary adenomas [22]. By means of immunohistochemistry, the labeling indices (LI) of p27kip1, PCNA and Ki-67 were calculated in both enlarged and non-enlarged adenomas. P27kip1 belongs to the kip1 family and regulates progression of G_1 to S phase in the cell cycle. In human corticotroph adenomas, a significant lower expression of p27kip1 was found compared with normal pituitary tissue [23]. In a p27kip1 knockdown mice model, mice developed pituitary adenomas with a threefold increase of the pars intermedia [24]. These results indicate a for a role in pituitary tumorigenesis. However, no significant difference in expression was found between canine pituitary adenomas and healthy pituitary tissue [22].

PCNA is a nuclear protein and its expression is correlated with cell proliferation [25]. It has been shown that the PCNA index is significantly higher in recurred human pituitary adenomas, compared with the non-recurrent group [25]. In canine pituitary adenomas, no significant difference in expression of PCNA was found between enlarged or non-enlarged tumors [22].

Ki-67 is a nuclear antigen expressed during the G_1, S, G_2, and M phases of the cell cycle but not in the G_0 phase [26]. In humans, Ki-67 is a widely investigated marker and is used to asses tumor proliferation. However, its association with tumor invasiveness, growth rate and recurrence has been described both positively and negatively [26]. As in humans, conflicting results were found regarding to Ki-67 expression in canine pituitary adenomas. In 2011, Ishino *et al* [27] showed a significant difference in Ki-67 and MCM7 expression between enlarged and non-enlarged canine pituitary tumors, with higher expression of both proteins in enlarged tumors. It is considered that differences in staining technique, experience in interpretation and variation in localization of Ki-67 in the cells have led to these conflicting results [26,27]. The high variation among published results makes Ki-67 not a suitable proliferation marker in pituitary tumors.

Minichromosome Maintenance (MCM) proteins play an important role in the initiation and regulation of DNA replication and MCM genes has been investigated in both human (MCM2) [28] and canine (MCM7) pituitary adenomas [27]. In 87% of the human corticotroph adenoma tissue immunoreactivity to the MCM2 antigen is found, while 20% of healthy pituitary tissue stained positively for MCM2 [28]. In canine tumors the MCM7 expression between enlarged and non-enlarged corticotroph tumors is investigated and a significant higher expression of the MCM7 gene is found in enlarged tumors [27]. Since MCM7 is responsible for cell proliferation, these results suggest that there is a higher cell proliferation in enlarged tumors resulting in a higher growth rate and thus, large tumors.

Recently, the expression and relevance of the transcription factors Sox2 and Pax7 in canine corticotroph pituitary adenomas is investigated (van Rijn *et al*, unpublished data). Pax7 is a member of the Pax transcription factor family and an essential regulator for the melanotroph fate. Expression of pax7 in the normal pituitary gland in mice is localized in the intermediate lobe [29]. Sox2 plays an important role in pituitary development [30] and is down regulated in the postnatal pituitary. However, a residual level of Sox2 positivity persists and these $Sox2^+$ cells show several stem

cell characteristics [31-33]. Injection of $Sox2^+$ cells do give rise to pituitary adenomas in transgenic mice [31], indicating for a role in tumorigenesis. However, no significant difference in labeling index is found between canine pituitary adenoma vs normal pituitary, enlarged vs non-enlarged adenoma and between recurrence vs no recurrence. Remarkably, expression of Pax7 was found in the anterior lobe of one healthy canine pituitary sample, despite its restricted expression in melanotroph cells. Besides, no expression of Pax7 was found in 4 out of 7 intermediate lobe adenomas.

Marker	Function	Results	Reference
Tpit	POMC cell lineage; Late differentiation of corticotrophs and melanotrophs	No mutations found in Tpit gene	Hanson, J *Domest Anim Endocrin* 2008:34(3) 217-22
LIF	POMC cell lineage; Differentiation of corticotrophs	No significance difference in expression between enlarged and non-enlarged adenomas	Hanson, J *Domest Anim Endocrin* 2010:38(4)260-71
EGFR	POMC cell lineage	Suppressed POMC expression after blocking EGFR in canine adenoma cell cultures with a tyrosin kinase inhibitor	Fukuoka, H *J Clin Invest* 2011:121(12) 4712-21
P27kip1	Cell cycle inhibitor	No significance difference in labeling indices between enlarged and non-enlarged tumors	Van Rijn, SJ *Domest Anim Endocrinol* 2010:38(4) 244-52
PCNA	Cell proliferation marker	No significance difference in labeling indices between enlarged and non-enlarged tumors	Van Rijn, SJ *Domest Anim Endocrinol* 2010:38(4) 244-52
Ki-67	Cell proliferation marker	No significance difference in labeling indices between enlarged and non-enlarged tumors Significance higher expression of Ki-67 in enlarged tumors	Van Rijn, SJ *Domest Anim Endocrinol* 2010:38(4) 244-52 Ishino, H *Domest Anim Endocrinol* 2011:41(4) 207-13
MCM7	Initiation and regulation of DNA replication	Significant higher expression of MCM7 in enlarged tumors	Ishino, H *Domest Anim Endocrinol* 2011:41(4) 207-13
Pax7	Essential regulator for the melanotroph fate	No significance difference in labeling indices between normal pituitary and pituitary adenoma	Van Rijn *et al*; unpublished
Sox2	Transcription factor essential in pituitary development	No significance difference in labeling indices between normal pituitary and pituitary adenoma	Van Rijn *et al*; unpublished

Table 3.2: Markers investigated in the canine pituitaries and pituitary adenomas.

3.6 (Cancer) stem cells in the pituitary gland

There are two types of stem cells, the embryonic stem cells and adult stem cells. Embryonic stem cells are totipotent and can give rise to all three germ layers and all differentiated cells, while adult stem cells are pluripotent or multipotent [34]. Pluripotent stem cells can differentiate into all cell types that make up the body and multipotent stem cells can generate more than one cell type but are more limited then pluripotent cells. Among the adult stem cells are the so-called cancer stem cells (CSCs). According to the cancer stem cell (CSC) model, it is hypothesized that tumors exist of phenotypically distinct populations of tumorigenic and non-tumorigenic cells. The tumorigenic cells give rise to the non-tumorigenic cells and are responsible for growth, maintenance and progression

of tumors, while non-tumorigenic cells do not contribute to tumor growth [35,36]. Injection of pancreatic CSCs in mice leads to aggressive tumor growth and metastasis [37]. Isolation and identification of CSCs will give insight in their self-renewal mechanisms and may help to understand the pathogenesis of pituitary adenomas. However, It is hard to isolate these cells because no specific stem cell markers are identified to isolate CSCs in the canine pituitary gland.

A promising approach to isolate CSCs is a technique called Fluorescence Activated Cell Sorting (FACS). This technique is based on the ability of stem cells to efflux the dye Hoechst 33342 by ATP-binding cassette (ABC) proteins, and a small cell fraction (side population, SP) can be separated from the majority of cells (main population, MP). The activity of the ABC transporters can be inhibited by verapamil, which can be used as a control. SP cells have been isolated from different tissues, and many times, the SP appeared to be enriched in stem cells.

Previously, our group successfully isolated the SP from healthy canine pituitaries [38]. FACS was done at the University of Leuven in the laboratory of Prof. H. Vankelecom where the technique of isolation and characterization of the SP from murine pituitary glands was established [39] and where SP was isolated from other neuroendocrine tissues as well, like adrenal glands and pancreatic tissues. After cell sorting the SP and MP of pituitary tissues was transported back to Utrecht and by quantitative PCR (qPCR) the expression of selected genes was characterized in the SP and MP. A significantly higher expression of the progenitor/stem cell markers CD34 and Thy1 was found in the SP, which indicated that the SP is enriched with stem cells. In the MP, expression of POMC and Lhx3 were significantly higher. There was no difference in the expression of CD133, Bmi-1, Axin2, Shh, Tpit, GH and PRL [38].

3.7 Cited references

[1] van Rijn SJ, Tryfonidou MA, Hanson JM, Penning LC, Meij BP. Stem cells in the canine pituitary gland and in pituitary adenomas. Vet Q 2013;33:217-24.

[2] Rijnberk A KH. Clinical endocrinology of dogs and cats. 2010.

[3] Hyttel P, Sinowatz F, Vejlsted M. Development of the central and perhipheral nervous system. In: Sinowatz F, editor. Essentials of domestic animal embryology: Saunders elsevier; 2010, p. 120-163.

[4] Zhu X, Gleiberman AS, Rosenfeld MG. Molecular physiology of pituitary development: signaling and transcriptional networks. Physiological reviews 2007;87:399-63.

[5] Panagiotakopoulos L, Neigh GN. Development of the HPA axis: where and when do sex differences manifest? Frontiers in neuroendocrinology 2014.

[6] Kent MS, Bommarito D, Feldman E, Theon AP. Survival, neurologic response, and prognostic factors in dogs with pituitary masses treated with radiation therapy and untreated dogs. Journal of Veterinary Internal Medicine 2007;21:1027-33.

[7] Meij B, Voorhout G, Rijnberk A. Progress in transsphenoidal hypophysectomy for treatment of pituitary-dependent hyperadrenocorticism in dogs and cats. Molecular and cellular endocrinology 2002;197:89-96.

[8] Neiger R, Ramsey I, O'Connor J, Hurley KJ, Mooney CT. Trilostane treatment of 78 dogs with pituitary-dependent hyperadrenocorticism. Vet Rec 2002;150:799-804.

[9] Barker EN, Campbell S, Tebb AJ, Neiger R, Herrtage ME, Reid SW et al. A comparison of the survival times of dogs treated with mitotane or trilostane for pituitary-dependent hyperadrenocorticism. Journal of Veterinary Internal Medicine 2005;19:810-5.

[10] den Hertog E, Braakman JC, Teske E, Kooistra HS, Rijnberk A. Results of non-selective adrenocorticolysis by o,p'DDD in 129 dogs with pituitary-dependent hyperadrenocorticism. The veterinary record 1999;144:12-7.

[11] Galac S, Buijtels JJ, Mol JA, Kooistra HS. Effects of trilostane on the pituitary-adrenocortical and renin-aldosterone axis in dogs with pituitary-dependent hypercortisolism. Vet J 2010;183:75-80.

[12] Braddock JA, Church DB, Robertson ID, Watson A, . Trilostane treatment in dogs with pituitary-dependent hyperadrenocorticism. Aust Vet J 2003;81:600-7.

[13] Ihle SL. Pituitary corticotroph macrotumors: diagnosis and treatment. The veterinary clinics of North America. Small animal practice 1997;27:287-97.

[14] Fornel de P, Delisle F, Devauchelle F, Rosenberg D.
Effects of radiotherapy on pituitary corticotroph macrotumors in dogs:
A retrospective study of 12 cases Can Vet J 2007;48:481-6.

[15] Hanson JM, Van 't Hoofd MM, Voorhout G, Teske E, Kooistra HS, Meij BP. Efficacy of transsphenoidal hypophysectomy in treatment of dogs with pituitary-dependent hyperadrenocorticism. Journal of Veterinary Internal Medicine 2005;19:687-94.

[16] Meij B. Hypophysectomy in dogs: a review. the veterinary quarterly 1999;21:134-41.

[17] Hanson JM, Teske E, Voorhout G, Galac S, Kooistra HS, Meij BP. Prognostic factors for outcome after transsphenoidal hypophysectomy in dogs with pituitary-dependent hyperadrenocorticism. J Neurosurg 2007;107:830-40.

[18] Hanson JM, Mol JA, Leegwater PAJ, Bilodeau S, Drouin J, Meij BP. Expression and mutation analysis of Tpit in the canine pituitary gland and corticotroph adenomas. Domest Anim Endocrinol 2008;34:217-22.

[19] Hanson JM, Mol JA, Meij BP. Expression of leukemia inhibitory factor and leukemia inhibitory factor receptor in the canine pituitary gland and corticotrope adenomas. Domestic animal endocrinology 2010;38:260-71.

[20] Theodoropoulou M, Arzberger T, Gruebler Y, Jaffrain-Rea ML, Schlegel J, Schaaf L et al. Expression of epidermal growth factor receptor in neoplastic pituitary cells: evidence for a role in corticotropinoma cells. The Journal of endocrinology 2004;183:385-94.

[21] Fukuoka H, Cooper O, Ben-Shlomo A, Mamelak A, Ren SG, Bruyette D et al. EGFR as a therapeutic target for human, canine, and mouse ACTH-secreting pituitary adenomas. The Journal of clinical investigation 2011;121:4712-21.

[22] van Rijn SJ, Grinwis GCM, Penning LC, Meij BP. Expression of Ki-67, PCNA, and p27kip1 in canine pituitary corticotroph adenomas. Domest Anim Endocrinol 2010;38:244-52.

[23] Lidhar K, Korbonits M, Jordan S, Khalimova Z, Kaltsas G, Lu X et al. Low expression of the cell cycle inhibitor p27Kip1 in normal corticotroph cells, corticotroph tumors, and malignant pituitary tumors. J Clin Endocrinol Metab 1999;84:3823-30.

[24] Oesterle EC, Chien WM, Campbell S, Nellimarla P, Fero ML. p27(Kip1) is required to maintain proliferative quiescence in the adult cochlea and pituitary. Cell Cycle 2011;10:1237-48.

[25] Hsu DW, Hakim F, Biller BM, de la Monte S, Zervas NT, Klibanski A et al. Significance of proliferating cell nuclear antigen index in predicting pituitary adenoma recurrence. J Neurosurg 1993;78:753-61.

[26] Salehi F, Agur A, Scheithauer BW, Kovacs K, Lloyd RV, Cusimano M. Ki-67 in pituitary neoplasms: a review- part I. Neurosurgery 2009;65:429-37.

[27] Ishino H, Hara Y, Takekoshi S, Teshima T, Teramoto A, Osamura RY et al. Ki-67 and minichrosome maintenance-7 (MCM7) expression in canine pituitary corticotroph adenomas. Domestic animal endocrinology 2011;41:207-13.

[28] Dallago CM, Barbosa-Coutinho LM, Ferreira NP, Meurer R, Pereira-Lima JF, Oliveira Mda C. Determination of cell proliferation using Mcm2 antigen and evaluation of apoptosis and TGF-beta1 expression in GH-secreting or clinically nonfunctioning pituitary adenomas. Endocrine pathology 2010;21:32-9.

[29] Budry L, Balsalobre A, Gauthier Y, Khetchoumian K, L'honoré A, Vallette S et al. The selector gene Pax7 dictates alternate pituitary cell fates through its pioneer action on chromatin remodeling. Genes & development 2012;26:2299-310.

[30] Alatzoglou KS, Kelberman D, Dattani MT. The role of SOX proteins in normal pituitary developement. J Endocrinol 2009;200:245-58.

[31] Andoniadou CL, Matsushima D, Mousavy Gharavy SN, Signore M, Mackintosh AI, Schaeffer M. Stem/Progenitor cells in the adult mouse pituitary support organ homeostasis and have tumor inducing potential. Cell Stem Cell 2013;13:433-45.

[32] Fauquier T, Rizzoti K, Dattani M, Lovell-Badge R, Robinson IC. SOX2-expressing progenitor cells generate all of the major cell types in the adult mouse pituitary gland. Proc Natl Acad Sci USA 2008;105:2907-12.

[33] Rizzoti K, Akiyama H, Lovell-Badge R. Mobilized adult pituitary stem cells contribute to endocrine regeneration in response to physiological demand. Cell Stem Cell 2013;13:419-32.

[34] Lloyd RV, Hardin H, Montemayor-Garcia C, Rotondo F, Syro LV, Horvath E et al. Stem cells and cancer stem-like cells in endocrine tissues. Endocrine pathology 2013;24:1-10.

[35] Magee JA, Piskounova E, Morrison SJ. Cancer stem cells: impact, heterogeneity, and uncertainty. Cancer Cell 2012;21:283-96.

[36] Meacham CE, Morrison SJ. Tumour heterogeneity and cancer cell plasticity. Nature 2013;501:328-37.

[37] Niess H, Camaj P, Renner A, Ischenko I, Zhao Y, Krebs S et al. Side population cells of pancreatic cancer show characteristics of cancer stem cells responsible for resistance and metastasis. Target Oncol 2014.

[38] van Rijn SJ, Gremeaux L, Riemers FM, Brinkhof B, Vankelecom H, Penning LC et al. Identification and characterisation of side population cells in the canine pituitary gland. Veterinary Journal 2012;192:476-82.

[39] Chen J, Hersmus N, Van Duppen V, Caesens P, Denef C, Vankelecom H. The adult pituitary contains a cell population displaying stem/progenitor cell and early-embryonic characteristics. Endocrinology 2005;146:3985-98.

4. Aims of the study

PDH is a common endocrinopathy is dogs and has a reported incidence of one to two cases per 1000 dogs per year [1]. It is hypothesized that CSCs are a subpopulation of cells that are responsible for growth, maintenance and progression of tumors [2]. Hypophysectomy and the availability of pituitary tumor tissue enable the isolation and characterization of these stem cells. In this way, new prognostic markers can be identified and ultimately, they could be used as new therapeutic targets, making therapy less invasive for the patient and more efficient against the disease. So far, no specific markers have been identified to isolate stem or progenitor cells in the canine pituitary gland.

The study discussed in chapter 3.6 forms the basis for this research project. This study shows that it is possible to isolate SP and MP cells from the canine pituitary gland, and that there is a difference in expression profile between both cell populations [3]. However, the study is done in another laboratory, and only canine healthy pituitary samples were sorted. The aim of the present study was (1) to optimize the FACS technique and to sort a side population of canine healthy pituitary and pituitary adenoma and (2) to investigate the best cell collection method after FACS analysis to obtain high quality RNA, required for quantitative gene expression analysis of the sorted cells.

Next to the optimization of the FACS technique, the role of PC2 and its association with the transcription factor Pax7 was investigated. This study is a continuation of the unpublished data of the Pax7/Sox2 study of van Rijn *et al*, done in our laboratory. In this study, the expression of the transcription factors Pax7 and Sox2 was investigated by means of immunohistochemistry in canine healthy pituitary and pituitary adenomas. Large variations were seen in Pax7 expression among the pituitary adenoma samples, and one healthy pituitary expressed Pax7. These results are in contrary to the findings of Budry *et al* [4], who reported Pax7 expression limited to the intermediate lobe of the pituitary in mice. Besides, an association of PC2 expression with Pax7 was found, and down regulation of the Pax7 gene resulted in a decrease of the gene expression of PC2, Drd2 and POMC in mice. The aim of this study was to investigate PC2 protein expression as well as gene expression of the genes Pax7, Sox2, PC2, Drd2, Thbs2 and POMC, to get more insight in the role of Pax7 and its associated proteins in canine pituitary tumorigenesis.

4.1 Cited references

[1] Willeberg P PW. Epidemiological aspects of clinical hyperadrenocorticism in dogs (canine Cushing's syndrome). 1982;18:717-24.

[2] Vankelecom H, Chen J. Pituitary stem cells: Where do we stand? Mol Cell Endocrinol 2014;385:2-17.

[3] van Rijn SJ, Gremeaux L, Riemers FM, Brinkhof B, Vankelecom H, Penning LC et al. Identification and characterisation of side population cells in the canine pituitary gland. Veterinary Journal 2012;192:476-82.

[4] Budry L, Balsalobre A, Gauthier Y, Khetchoumian K, L'honoré A, Vallette S et al. The selector gene Pax7 dictates alternate pituitary cell fates through its pioneer action on chromatin remodeling. Genes & development 2012;26:2299-310.

5. Optimization of the FACS technique and isolation of a side population in canine healthy pituitary and pituitary adenoma

5.1 Introduction

In 1996, Goodell *et al* [1] isolated for the first time a side population from murine bone marrow cells by means of FACS analysis. The sorted SP cells expressed the stem cell marker Sca-1 and comprised 0.1% of the bone marrow. The technique is based on the ability of SP cells to excrete the dye Hoechst 33342 by ATP-binding cassette sub-family G member 2 (ABCG2 transporters). The difference in Hoechst fluorescence emission between SP and MP cells can be detected using FACS analysis. When viewed in a dot plot the SP cells are low in both blue emission and red emission and they appear to be in the far left of the FACS plot (figure 5.1A). The activity of the ABCG2 transporters can be blocked by Verapamil and used as a control to set the sorting gate(figure 5.1B).

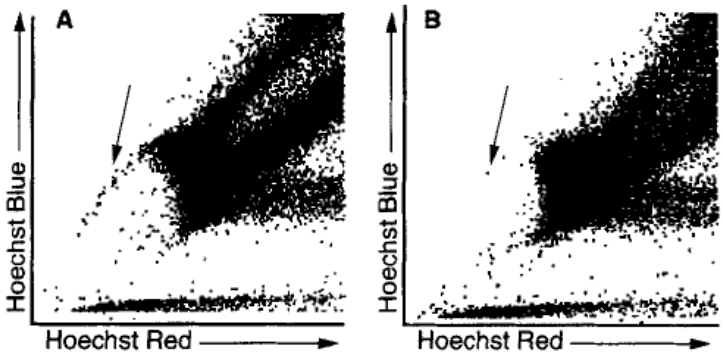


figure 5.1: Goodell *et al* isolated for the first time a side population from murine bone marrow cells. (A) whole bone marrow stained with Hoechst 33342. The arrow points the SP cells. (B) Inhibition of ABCG2 transporter by verapamil. *Goodell et al J Exp Med. 1996:183(4):1797-806*.

Today, the SP is identified in many tissues and tumors. In healthy tissue, SP proportions differ from 0.1% in bone marrow cells [1], up to 15.1% in whole brain cells [2]. In tumors, the smallest and largest SP fractions are found in prostate cancer (0.5%) [3] and mesenchymal neoplasm (10%) respectively [4]. Wu *et al* found a correlation between the proportion of the SP and aggressiveness of mesenchymal neoplasm [4] and it has been reported that the SP cells in multi-drug resistant canine lymphoma do not respond to inhibition with verapamil [5]. These reports indicate that there is a high variation in quantity and behavior of the SP fraction among different tumors, and there is a potential for SP isolation to support in prognosis and to improve cancer therapies.

Chen *et al* [6] successfully isolated a side population from the murine pituitary gland and the SP comprised of 1.7% of the total cell number. A subset of the SP cells expressed Sca-1 and it was found that not the Sca-1^{high}, but the Sca-1^{low} cells had progenitor characteristics [7]. In 2011, our research group isolated and characterized a SP from the healthy canine pituitary gland [8]**.** The cell dispersion protocol and FACS analysis was done at the University of Leuven in the laboratory of Prof. H. Vankelecom, where the technique of isolation and characterization of the SP from murine pituitary

glands was established [6]. Until now, only in healthy pituitary tissue the SP has been isolated. The aim of the present study was to optimize the cell dispersion protocol and FACS technique at Utrecht University and to sort a side population of canine healthy pituitaries and pituitary adenomas.

5.2 Materials & Methods

5.2.1 Experimental design

Canine healthy pituitary, canine pituitary adenoma and feline pituitary adenoma samples were collected and stored in liquid nitrogen at -190˚C or used fresh for cell digestion. To investigate how cell count and percentage of life cells is influenced, different digestion methods and media were used. Cells were sorted using two different FACS machines. Total life cell count, percentage life cells, appearance of a SP, inhibition by verapamil and the number of sorted cells were compared among the samples.

5.2.2 Sample collection

Canine corticotroph adenoma samples were collected between 2010 and 2014 from patients with PDH treated by transsphenoidal hypophysectomy in our clinic. A representative part of the removed tumor was fixed in 4% methanol-formaldehyde required for the pathological diagnosis, and the remainder was cut into small blocks, collected in Leibowitz (GIBCO, Invitrogen) +10% FCS (PAA laboratories)+10% DMSO (Sigma-Aldrich) and gradually frozen in a Mr. Frosty before storage in liquid nitrogen at -190˚C. Feline corticotroph adenoma samples were obtained from patients with acromegaly treated by transsphenoidal hypophysectomy. Samples were collected in D-MEM(4500 mg/L glucose containing l-glutamine and pyruvate) (GIBCO, Invitrogen) enriched with 1% penicillin streptomycin (GIBCO), 25mM HEPES (Sigma-Aldrich) and 3% bovine serum albumin (BSA)(Sigma-Aldrich), and freshly processed. All adenoma samples were collected after consent of the patient owners. Healthy pituitary samples were obtained from experimental dogs sacrificed in non-related experiments between 2013 and 2014. Immediately after the dogs were euthanized the skull was opened and the pituitary gland removed. The neurointermediate lobe was dissected free from the anterior lobe, and the anterior lobe was either digested immediately or stored as described above.

5.2.3 Cell dispersion

Different protocols were used for the dispersion of adenoma and healthy pituitary samples. Healthy pituitaries were digested using trypsin and adenoma samples were digested using collagenase. One healthy canine pituitary was digested using the adenoma protocol, and one feline pituitary adenoma was digested by trypsinization. Healthy pituitary samples were digested using a modified protocol of Denef [9]. Samples were thawed in a 37°C water bath and cut into small blocks using two surgical blades nr 11. After two wash steps with D-MEM(4500 mg/L glucose containing l-glutamine and pyruvate) enriched with 1% penicillin streptomycin, 25mM HEPES and 3% BSA, the tissue blocks were incubated with 2 ml of 2.5 w/v% trypsin solution (GIBCO) at 37°C. After 15 minutes of incubation, enriched D-MEM with 4µg DNase (Sigma-Aldrich) was added for 1 minute. The supernatant was replaced by 2 ml of EDTA-solution(HBSS, GIBCO) enriched with 1% penicillin streptomycin, 20 mM HEPES, 0.3% BSA and 2.5 mM EDTA (Sigma-Aldrich) and incubated for 5 minutes at 37°C to block the trypsin reaction. The tissue blocks were washed three times in HBSS solution enriched with 1% penicillin streptomycin, 20mM HEPES and 0.3% BSA. For the first wash

step 10% (FCS)(PAA laboratories) was added. To disperse tissue clumps a Wheaton potter and pestle was used. The cell suspension was transferred to a tube containing 4,5 ml enriched D-MEM solution and 5µg DNase and filtered through a nylon mesh (70µm). A 3% BSA in enriched D-MEM was added at the bottom of the tube and the tube was centrifuged (5 minutes; 1500 rpm; RT) to remove cell debris. The cell pellet was resuspended in either medium 1A or medium 2A. Medium 1A consisted of one liter DMEM 1:1/F12 (GIBCO) containing 5 mg transferrin (Invitrogen), 5 mg Insulin (Sigma-Aldrich), 1% penicillin streptomycin, 1 mg catalase (Sigma-Aldrich), 1 g $NaHCO_3$ (Merck, Germany), 600µl EtOH 96% (Merck, Germany), 0.6µm Fe^{2+}/Fe^{3+} and 0.5% BSA [10]. Medium 2A consisted of DMEM-Glutamax enriched with 2% FCS and 10 mM Hepes. Cells were counted using a cell counter (Bio-Rad TC10™) and diluted to 1 million cells/ml.

To disperse pituitary adenoma cells, tissue was incubated with collagenase IV (GIBCO) (in α-MEM 1 mg/ml, GIBCO) for 90 minutes at 37°C and pipetted up and down every 20 minutes. After incubation, the suspension was filtered through a 40 µm nylon mesh and collected in a tube containing HBSS enriched with 20% FCS, to inactivate collagenase. Remaining pieces were dispersed with HBSS + 20% FCS using a pipet and filtered. HBSS + 20% FCS was added until the collagenase solution was twofold diluted and the cell suspension was washed two times in HBSS + 20% FCS. The cell pellet was resuspended in either medium 1A or 2A, counted using a cell counter and diluted to 1 million cells/ml.

5.2.4 Hoechst staining

Hoechst staining was performed directly after cell dispersion, apart from the fresh samples. Fresh samples were digested and kept overnight at 37°C in a 50 ml tube containing 12.5 ml DMEM(4500 mg/L glucose containing l-glutamine and pyruvate) +10% FCS +1% penicillin streptomycin, previously to Hoechst staining. For the Hoechst staining protocol, cell density was adjusted to 1 million cells/ml and control samples were incubated with 100µM Verapamil (Centrafarm) for 20 minutes at 37°C. Hoechst 33342 (2.5µg/ml)(Sigma-Aldrich) was added to all samples and incubated for 90 minutes at 37°C. After 45 minutes of incubation, samples were gently mixed. To stop the reaction, samples were put on ice, diluted twofold in ice cold medium 1A or 2A and centrifuged at 4°C (1500 rpm; 5 minutes). Cell pellets were resuspended in ice-cold sorting solution. Two different sorting solutions were used, 1B and 2B. Sorting solution 1B was defined by our laboratory in Utrecht and consisted of HBSS enriched with 2% FCS, 2µg/ml propidium iodide (PI)(Sigma-Aldrich), 0.4% DNase and 0.025% EDTA. Sorting solution 2B was composed according to the Hoechst staining protocol from Leuven and consisted of HBSS, 2% FCS and 2µg/ml PI. All steps were protected from light and samples were kept on ice until FACS analysis.

5.2.5 FACS analysis

Flow cytometry was carried out at the Hubrecht Institute (Utrecht) using a FACSaria II (SORP) and at the department of Immunology at the Faculty of Veterinary Medicine (Utrecht University) using a BD Influx. The Hoechst dye fluorescence was measured at two wavelengths. A plot was drawn of Hoechst-red vs Hoechst-blue and single cells were selected, followed by scatter gating and live gating, to exclude dead cells. PI positive cells were seen on the far right of the Hoechst-red. The verapamil inhibited control was used to adjust the SP sorting gate.

5.2.6 Statistical analysis

Statistical analysis was performed in IBM SPSS (22). To test for normal distribution, a Shapiro-Wilk test was used. To investigate whether there was a significant difference between fresh and frozen samples, between medium 1A/1B and 2A/2B, and between the SP fractions, a Mann-Whitney U test was performed. Correlation between percentage life cells and preservation of the samples was calculated using a Spearman's Rho test. Significance was set at $P < 0.05$.

5.3 Results

5.3.1 Cell digestion

In table 5.1, data of the samples and cell count are summarized. In total, 19 canine pituitary adenomas, 3 feline pituitary adenomas and 24 healthy pituitaries were digested. Total life cell count ranged from 10,100 to 6.87×10^6 for canine adenoma samples, from 76,000 to 580,000 for the feline adenoma samples and from 30,900 to 2.46×10^6 cells for the normal pituitary gland samples, with a median of 642,000, 149,000 and 265,500 cells respectively. In figure 5.2, total life cell count of the samples is displayed, with different bars for fresh and frozen samples (figure 5.2A), and for the different kinds of media used (figure 5.2B).

Percentage of life cells ranged from 5% to 44% for canine adenoma samples, from 2% to 5% for the feline adenoma samples and from 11% to 43% cells for the normal pituitary gland samples, with a median of 26%, 4% and 24% respectively. Figures 5.3A and B display percentage life cells and its association with the different samples and protocols used. One feline pituitary adenoma was digested according to the healthy pituitary protocol, and total life cell count was 149.000 with 4% life cells. One healthy pituitary was kept overnight at 4°C in D-MEM(4500 mg/L glucose containing l-glutamine and pyruvate) enriched with 1% penicillin streptomycin, 25mM HEPES and 3% BSA and digested with collagenase according to the adenoma protocol. Total life cell count was 30,300 with 24% life cells.

Table 5.1: Summary of the used protocol, total life cell count and life percentage of the healthy pituitary and adenoma samples used in this study.

Sample number	Tissue	Species	Sample collection	Fresh/Frozen	Digestion	Media	Total life count	%life
1	adenoom	Canine	2-2-2010	Frozen	Collagenase	1A/1B	370.000	26
2	adenoom	Canine	6-1-2014	Frozen	Collagenase	1A/1B	6.200.000	41
3	hypofsye	Canine	2-4-2014	Frozen	Trypsine	1A/1B	455.000	31
4	hypofyse	Canine	25-3-2014	Frozen	Trypsine	1A/1B	252.000	24
5	hypofyse	Canine	30-4-2014	Frozen	Collagenase	1A/1B	30.300	24
6	Adenoom	Canine	6-5-2014	Frozen	Collagenase	1A/1B	1.040.000	15
7	hypofyse	Canine	7-5-2014	Frozen	Trypsine	1A/1B	482.700	28
8	hypofyse	Canine	22-4-2014	Frozen	Trypsine	1A/1B	248.000	21
9	hypofyse	Canine	22-4-2014	Frozen	Trypsine	1A/1B	80.900	29
10	hypofyse	Canine	22-4-2014	Frozen	Trypsine	1A/1B	167.000	14
11	hypofyse	Canine	22-4-2014	Frozen	Trypsine	1A/1B	177.000	22
12	adenoom	Canine	1-6-2010	Frozen	Collagenase	1A/1B	814.000	29
13	adenoom	Canine	8-6-2010	Frozen	Collagenase	1A/1B	1.200.000	19
14	adenoom	Canine	5-3-2013	Frozen	Collagenase	1A/1B	566.000	28
15	adenoom	Canine	4-3-2013	Frozen	Collagenase	1A/1B	4.220.000	39
16	adenoom	Canine	11-3-2014	Frozen	Collagenase	1A/1B	46.000	21
17	adenoom	Canine	9-2-2010	Frozen	Collagenase	1A/1B	460.000	26
18	adenoom	Canine	27-5-2014	Frozen	Collagenase	1A/1B	971.000	23
19	adenoom	Canine	9-3-2010	Frozen	Collagenase	1A/1B	642.000	33
20	adenoom	Canine	1-10-2013	Frozen	Collagenase	1A/1B	10.100	6
21	adenoom	Canine	11-11-2013	Frozen	Collagenase	1A/1B	6.870.000	41
22	adenoom	Canine	12-11-2013	Frozen	Collagenase	1A/1B	20.200	5
23	adenoom	Canine	15-2-2011	Frozen	Collagenase	1A/1B	4.640.000	44
24	adenoom	Canine	12-3-2013	Frozen	Collagenase	1A/1B	154.000	8
25	adenoom	Canine	26-1-2010	Frozen	Collagenase	1A/1B	743.000	10
26	adenoom	Canine	25-3-2014	Frozen	Collagenase	1A/1B	152.00	33
27	adenoom	Canine	2-2-2010	Frozen	Collagenase	1A/1B	75800	21
28	hypofyse	Canine	22-4-2014	Frozen	Trypsin	1A/1B	131.500	22
29	hypofyse	Canine	22-4-2014	Frozen	Trypsin	1A/1B	258.000	24
30	hypofyse	Canine	22-4-2014	Frozen	Trypsin	1A/1B	113.500	33
31	hypofyse	Canine	22-4-2014	Frozen	Trypsin	1A/1B	273.000	25
32	hypofyse	Canine	23-4-2014	Frozen	Trypsin	1A/1B	245.000	12
33	hypofyse	Canine	16-4-2014	Frozen	Trypsin	1A/1B	1.020.000	30
34	hypofyse	Canine	9-4-2014	Frozen	Trypsin	1A/1B	245.000	31
35	hypofyse	Canine	14-4-2014	Frozen	Trypsin	1A/1B	290.500	25
36	hypofyse	Canine	7-4-2014	Frozen	Trypsin	1A/1B	2.460.000	43
37	hypofyse	Canine	26-3-2013	Frozen	Trypsin	2A/2B	900.000	31
38	hypofyse	Canine	16-7-2014	Frozen	Trypsin	2A/2B	1.060.000	17
39	hypofyse	Canine	24-7-2010	Frozen	Trypsin	2A/2B	440.000	11
40	hypofyse	Canine	19-8-2014	Fresh	Trypsin	2A/2B	201.000	19
41	Adenoom	Feline	10-9-2014	Fresh	Collagenase	2A/2B	580.000	5
42	Hypofyse	Canine	10-9-2014	Fresh	Trypsin	2A/2B	341.000	14
43	hypofyse	Canine	15-9-2014	Fresh	Trypsin	2A/2B	351500	19
44	Adenoom	Feline	17-9-2014	Fresh	Trypsin	2A/2B	149000	4
45	adenoom	Feline	24-9-2014	Fresh	Collagenase	2A/2B	76000	2
46	hypofyse	Canine	24-9-2014	Fresh	Trypsin	2A/2B	467500	19

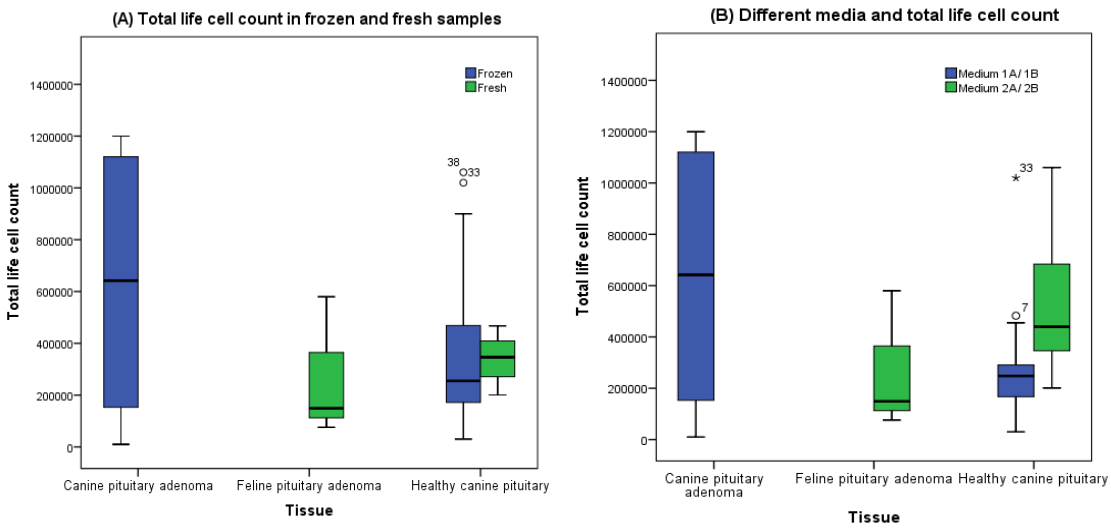


Figure 5.2: Different protocols were used to investigate the best protocol in order to obtain a high total life cell count. In both graphs, sample number 2, 15, 21, 23 and 36 are not displayed, but are used in the calculation of the median. **(A)** Total life cell count was measured in freshly digested and frozen samples. Frozen canine pituitary adenoma (n=19), fresh feline pituitary adenoma (n=3), frozen healthy canine pituitary (n=20) and fresh canine healthy pituitary (n=4). **(B)** Two kinds of media were used in the cell digestion protocol, medium 1A/1B and medium 2A/2B. Canine pituitary adenoma media 1A/1B (n=19), Feline pituitary adenoma media 2A/2B (n=3), healthy canine pituitary media 1A/1B (n=17), healthy canine pituitary media 2A/2B (n=7).

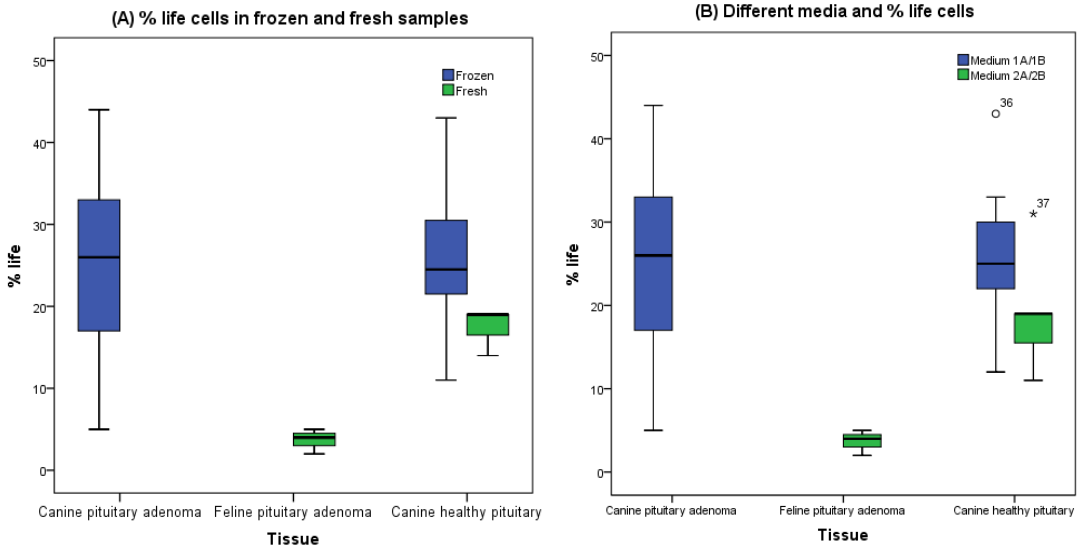


Figure 5.3: Different protocols were used to investigate the best protocol in order to obtain a percentage of life cells. **(A)** %life cells was calculated in freshly digested and frozen samples. Frozen canine pituitary adenoma (n=19), fresh feline pituitary adenoma (n=3), frozen healthy canine pituitary (n=20) and fresh canine pituitary adenoma (n=4). **(B)** Two kind of media are used in the cell digestion protocol, medium 1A/1B and medium 2A/2B. Canine pituitary adenoma media 1A/1B (n=19), Feline pituitary adenoma media 2A/2B (n=3), healthy canine pituitary media 1A/1B (n=17), healthy canine pituitary media 2A/2B (n=7).

Only for the healthy pituitary samples, fresh and frozen samples, as well as the two different kinds of media were used. In table 5.2, mean total life cell count, percentage life cells and statistic outcome of the canine healthy pituitary samples are depicted. A significant difference was found between the fresh and frozen samples, with a higher percentage life cells in the frozen samples (P = 0.048). Cells digested using medium 1A/1B had a significantly better survival (P = 0.022), compared with cells digested with medium 2A/2B.

Healthy pituitary and pituitary adenoma samples were preserved in nitrogen or directly digested, as described in section 5.2.2. Healthy pituitary samples were stored for 0 to 49 months, with a median of 1 month. Pituitary adenoma samples were stored for 0 to 53 months, with a median of 11.5 months. In figure 5.4, the association between cell survival and preservation is illustrated. All samples were included into the table, both feline and canine pituitary adenomas, fresh (0 months) and frozen samples, and samples digested with the different kinds of media. Percentage life cells and duration of preservation of the samples, was not correlated (P = 0.075, correlation coefficient (CC) = 0.265).

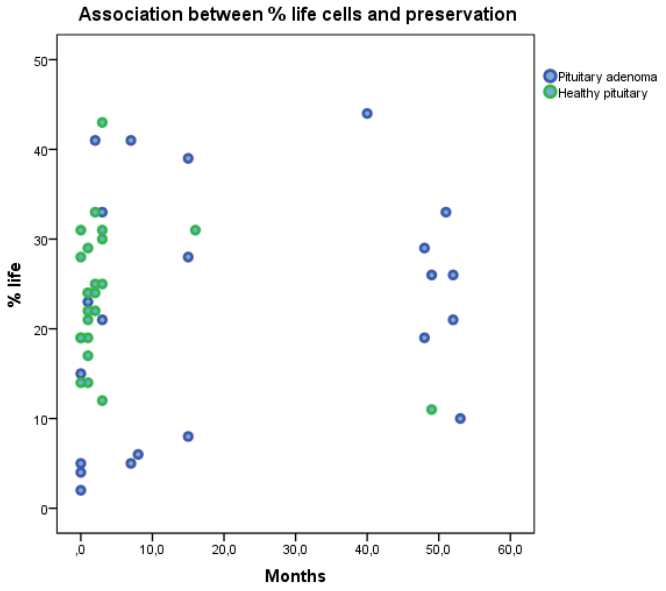


Figure 5.4: Association between percentage life cells and preservation. All samples were included in the figure. Percentage life cells and duration of preservation (months) were not correlated (P = 0.075, CC = 0.265). Healthy pituitary (n=24), pituitary adenoma (n=22).

	Mean ±standard deviation		*P*-value
	Frozen	**Fresh**	
Total life cell count	466,470±124,337	340,259±54,551	0.588
% life	24.85±1.73	17.75±1.25	0.048
	Medium 1A/1B	**Medium 2A/2B**	
Total life cell count	407,611±139,259	537,285±120,045	0.053
% life	25.76±1.74	18.57±2.37	0.022

Table 5.2: Mean total life cell count, percentage life cells and statistics of the canine healthy pituitary samples. *difference is significant at the 0.05 level (2-tailed)

5.3.2 FACS analysis

FACS analysis was performed on a FACSAria II (SORP) and on a BD influx FACS machine. The specifications of both FACS machines are listed in table 5.3. The FACSAria II (SORP) was equipped with a 10mW Violet (407 nm) solid-state laser. Blue fluorescence emission was measured using a 450/40 nm band pass (BP) filter, and Hoechst red was collected thought a 605 nm long pass (LP) filter. Using the BD Influx, Hoechst blue was collected through a 424/40 nm BP filter and Hoechst red fluorescence emission was measured through a 670/30 nm BP filter. Although Hoechst emission profiles vary between the two instruments (figure 5.5), the side population could be clearly identified with both FACS machines.

In total, 39 of the 46 samples were sorted by means of FACS. All canine pituitary adenomas (17) and 14 healthy canine pituitaries were sorted using a FACSAria II (SORP). All feline pituitary adenomas (3) and 5 healthy pituitaries were sorted using a BD Influx. In total, 7 samples were digested but not analyzed. Five samples were used to optimize the digestion protocol and to set the gating for FACS analysis and of two healthy pituitary samples, total life cell count was too low to perform FACS analysis.

FACS machine	FACSAria II (SORP)	BD Influx
Location	Hubrecht institution, Utrecht	Faculty of Veterinary Medicine, department of Immunology, Utrecht University
Laser wavelength (nm)	407	355
Hoechst blue filter (nm)	450 /40 BP	424/44 BP
Hoechst red filter (nm)	605 LP	670/30 BP
Pressure (PSI)	19	25
Nozzle (micron)	100	100
Flow rate (events/second)	200-1000	600-2000

Table 5.3: Specifications of the two FACS machines, used for cell sorting. BP, band pass; LP, long pass

After the cell digestion protocol, but before incubation with Hoechst 33342, the total number of life cells was counted and percentage life cells was calculated. Of the samples sorted with the FACSAria II (SORP), percentage life cells during FACS analysis was calculated. Calculated cell survival differed between both measurements. In 36 samples, percentage life cells calculated during FACS analysis was decreased compared with the percentage of life cells after cell digestion and a decrease from 25±10% SD to 12.8±5% SD was seen. For 3 samples, an increased cell survival was calculated during FACS analysis, with an increase in percentage life cells from 19.3± 11.5% SD to 43.6± 30.7% SD.

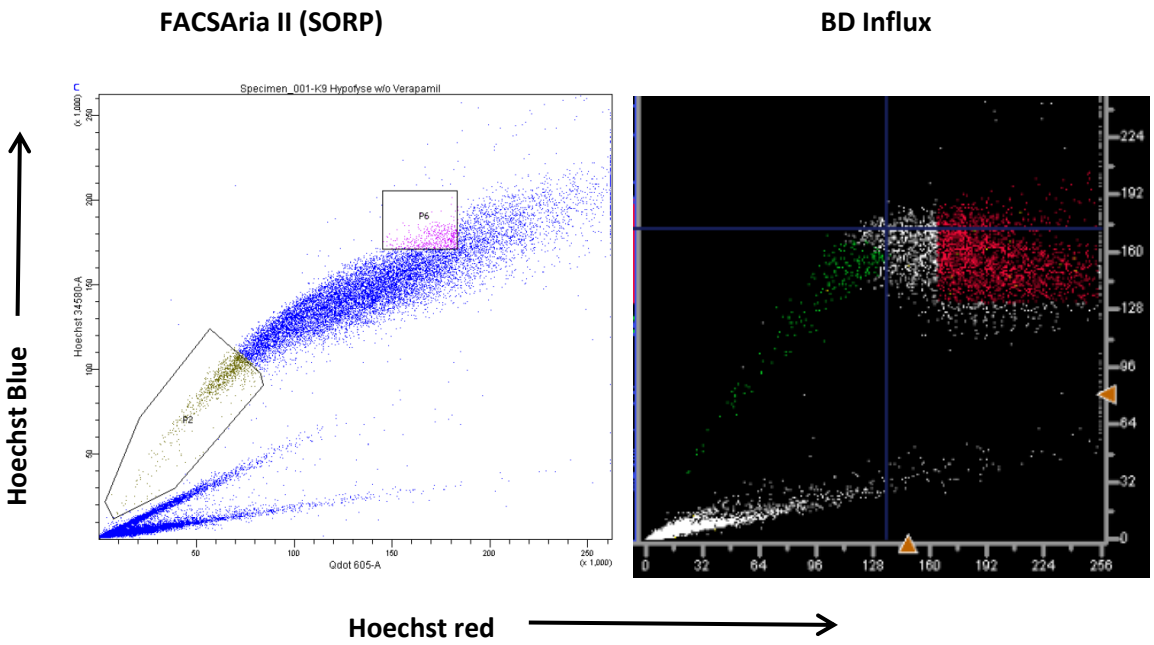


Figure 5.5: Comparison of SP profiles between flow cytometers. Although there are subtle differences between the plots, the SP in clearly visible in both cases. Two different healthy canine pituitary samples were used.

An Hoechst^{low} SP was sorted from an Hoechst^{high} MP and samples blocked with verapamil did not have an Hoechst^{low} population. Debris is Hoechst negative and appear towards the origin, with a low Hoechst blue fluorescence emission. Figure 5.6A shows the results of FACS analysis of an healthy canine pituitary sample. The SP cells are outlined as the green cells and they appear to be in the far left of the plot, due to the Hoechst dye efflux. Figure 5.6B shows a sample of the same pituitary, but incubated for 20 minutes with verapamil. Hoechst dye efflux is reduced and less green cells are seen in the plot. In figure 5.6C and D, FACS analysis of a canine pituitary adenoma with and without verapamil is showed. All samples showed a reaction to verapamil, except for 3 adenoma samples (number 16, 21 and 26) and 2 pituitary samples (number 37 and 42). Figure 5.5E and F shows the results of FACS analysis of sample number 26.

Of the samples analyzed on the FACSAria II (SORP) that did show a response to verapamil, mean percentage SP was calculated. After incubation with verapamil, Hoechst dye efflux was significantly reduced in both healthy pituitary and pituitary adenoma samples. Mean SP fraction was 0.70±0.96% in healthy pituitary samples, and decreased to 0.06±0.1% (P<0,01) in the control samples. In pituitary adenomas, a SP fraction of 1.01±1.36% significantly reduced to 0.23±0.35% (P = 0.015). No significant difference was found between SP fraction in healthy pituitary and in pituitary adenoma (P = 0.273).

Variances were seen in the SP fractions of both healthy pituitary and pituitary adenoma samples, measured on the FACSAria II (SORP). In healthy pituitary samples, SP fraction ranged from 0.1 to 2.9% and in pituitary adenomas the fraction SP cells ranged from 0.1 – 4.1%. Proportion SP cells and percentage life cells in the samples was significantly correlated (P = 0.040, CC = 0.405).

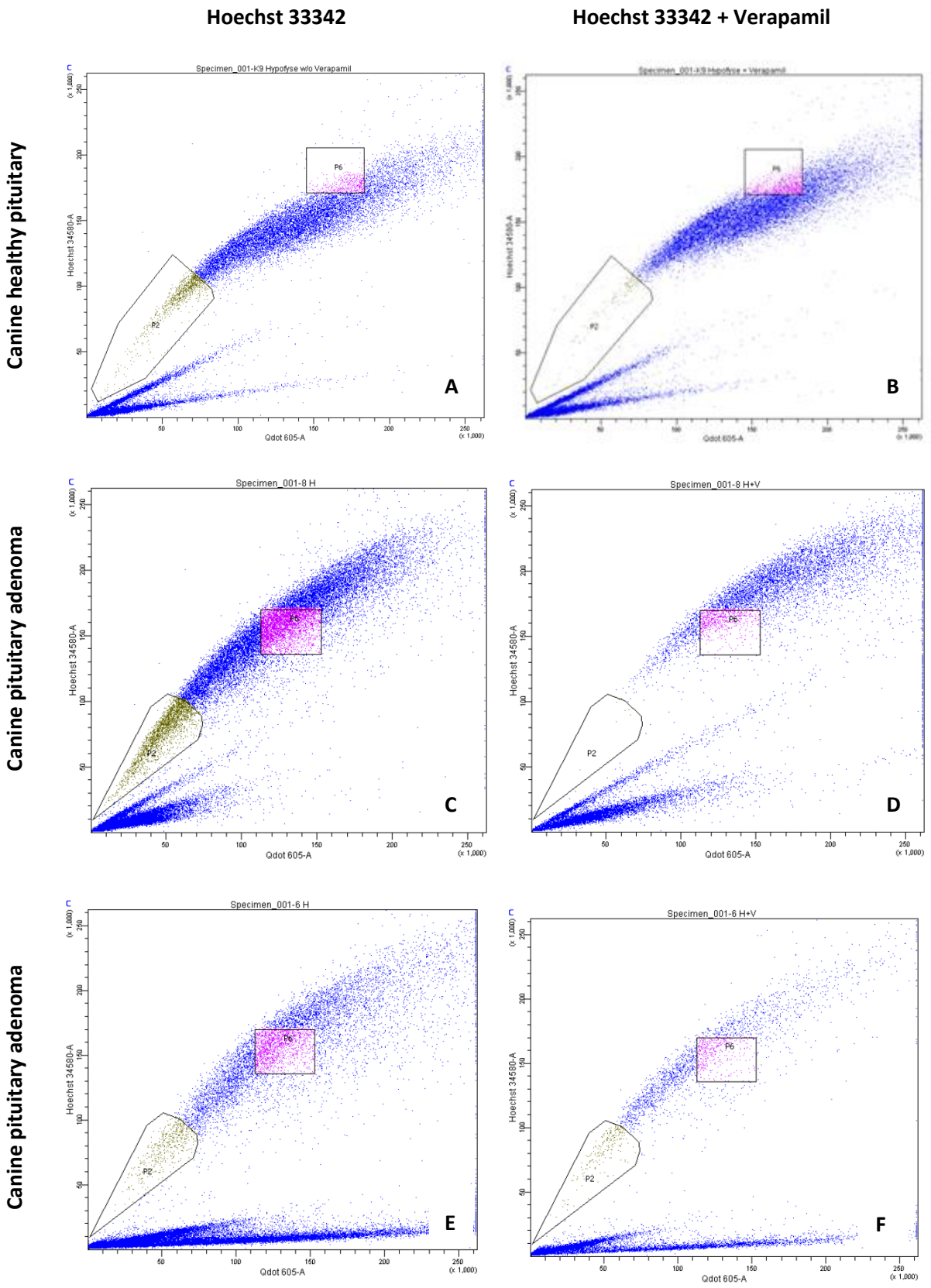


Figure 5.6: Fluorescent activated cell sorting analysis, performed on a FACSAria II (SORP), of canine healthy pituitary and canine pituitary adenoma after incubation with the dye Hoechst 33342. Samples were incubated with (B,D,F) and without (A,C,E) verapamil. The SP comprises 0.4% (A), 0.1% (B), 0.4% (C), 0.0% (D), 0.1% (E) and 0.1% (F) respectively.

5.4 Discussion

Since the establishment of a FACS technique to sort SP cells from murine bone marrow cells [1], the SP is sorted from many tissues and tumors. In this study, we optimized the cell digestion protocol and FACS technique for canine healthy pituitary and pituitary adenomas. The results suggest that it is possible to sort a SP from canine pituitary adenomas. However, sorting of the correct fraction SP cells is a precise and amendable technique.

Denef *et al* [9] described a protocol for the digestion of murine pituitary tissue with a cell survival of up to 95%. In our study, cell survival of 2 to 44% was found, with the lowest survival in fresh digested feline pituitary adenomas (2-5%). Previously, FACS analysis was performed on canine healthy pituitaries and a modification of the protocol of Denef *et al* was used [8]. In total, 6 healthy pituitaries were harvested and immediately digested, and a yield of 3.5-6 $x10^6$ cells was obtained [8]. In the present study, we used the same protocol for cell digestion, but with different sample collection methods. Samples were frozen and stored in liquid nitrogen previously to cell digestion and mean total life cell count was 466,470 ±124,337 SD in canine healthy pituitaries. To test whether the reduced amount of life cells was due to freezing of the samples, fresh samples were digested. A statistically higher cell survival in frozen canine healthy pituitary samples was found, compared with fresh samples (Table 5.2). This remarkable observation may be explained by a change of media. Apart from four, all frozen samples were digested with the use of media 1A/1B as described previously [8,9]. All fresh samples were digested with the use of media 2A/2B. As shown in table 5.2, cell survival was significantly higher in samples processed with the use of media 1A/1B compared with media 2A/2B. However, cells were resuspended in the different media and cell count was performed within 5 minutes. It is unknown in what extent the media may have influenced cell count. Since two parameters were changed simultaneously, and less fresh (n=4) than frozen (n=20) samples were digested, it is difficult to explain whether the higher cell survival in frozen samples is due to preservation, the change of media or due to incorrect conclusions because of the difference in group size.

In canine pituitary adenomas, total life cell count ranged from 10,100 to $6.87x10^6$. It was not possible to compare total life cell count among the adenoma samples due to the high variance in dog breed and tumor size. Cell density and weight of the collected tumorous tissue was not measured and therefore, it is better to use cell survival as a parameter to compare adenoma samples. There was no correlation found between the duration of preservation and cell survival in both healthy pituitary and adenoma samples (figure 5.4). The effect of freezing on cell survival in pituitary adenomas, compared with fresh processing of the samples, could not be analyzed. All canine pituitary adenomas were frozen, whereas all feline pituitary adenomas were processed immediately after hypophysectomy. Feline pituitary adenomas were obtained from patients suffering from acromegaly, caused by hyperplasia of GH producing somatotropes. The use of samples of a different species and cell type without modifying the protocol, may be the cause of the low cell survival in feline adenoma samples.

In this study, two different instruments were used to identify $Hoechst^{low}$ SP cells. In total, 31 samples were analyzed using a FACSAria II (SORP) and 8 samples were analyzed using a BD influx. Simpson *et al* [11] analyzed Hoechst SP detection with the use of different excitation sources and instruments. Despite the use of different laser types, collection filters and wavelengths, a SP could be detected on

all instruments. However, differences in appearance of the Hoechst profiles were seen [11]. These results are comparable with our findings. Measuring blue and red fluorescence emission with the FACSAria II (SORP), the cell population formed a linear band, with the SP cells in the far left of the dot plot. When red and blue fluorescence emission were detected with a BD influx, the cell population was less spread out, not linear, and the SP cells appeared to form a tail at the left of the cell population (figure 5.5). Simpson *et al* analyzed the same Hoechst stained murine bone marrow preparation on three different FACS machines [11]. However, calculated SP fractions were not compared among the different instruments. Since it was not investigated whether SP fractions differ when measured with different FACS machines, it is not preferred to compare the fraction SP cells in our samples measured on the FACSAria II (SORP) or BD Influx.

Hoechst red and blue fluorescence emission was not the same in all samples and thus, the location of the cell population in the dot plot differed between both healthy pituitary and adenoma samples (figure 5.7). The use of patient material instead of cell lines, may have influenced the location of the cell population. An excess of Hoechst is needed to stain all the DNA in all cells, but Hoechst is toxic and high concentrations will lead to cell death [12]. The correct Hoechst concentration has to be assessed for each cell type and in this study a concentration of 2.5 µg/ml was used, as described previously for healthy pituitary cells by van Rijn *et al* [8]. DNA content may differ between healthy pituitary and adenoma samples, as well as among adenoma samples. When a lesser amount of Hoechst is added relatively to DNA content, less Hoechst fluoresce will be detected which causes a shift of the cell population. No information is available about the DNA content of the cells in the samples used for this study.

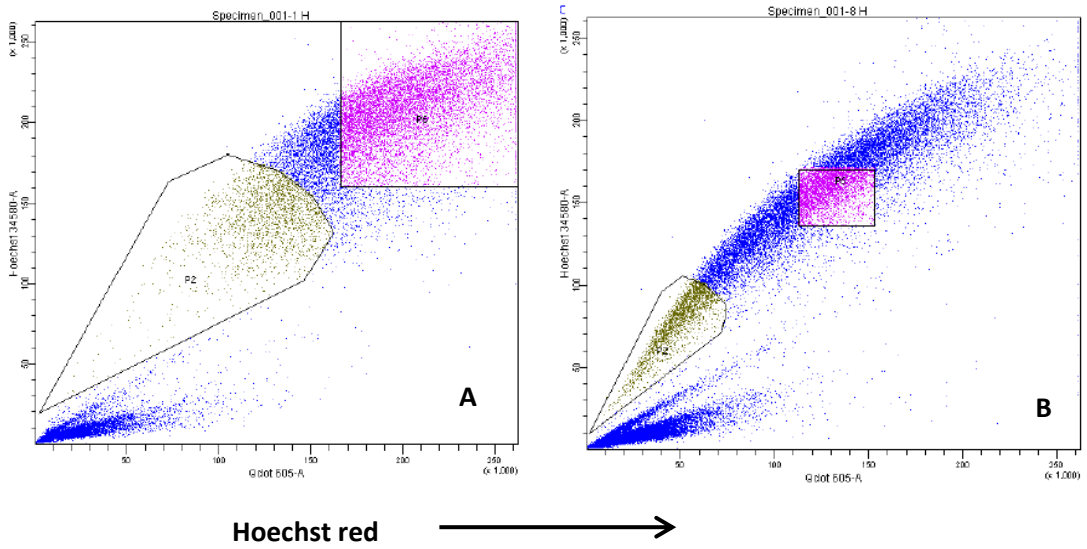


Figure 5.7: Differences in appearence of cell population and SP in two adenoma samples incubated with Hoechst, with a shift of the cell population towards the left depicted in dot plot B. (A) Mean red excitation is 175,000. (B) Mean red excitation is 130,000.

Three pituitary adenoma samples did not show inhibition by verapamil (figure 5.6 E, F). Verapamil blocks the ABCG2 transporters expressed in the SP cells, that actively remove Hoechst. Kim *et al* [5] investigated the SP in canine lymphoma cell lines and from clinical cases of canine lymphoma, and treatment with verapamil did not affected the SP proportion. Different concentrations of verapamil were investigated (25, 50 and 100 µM), as well as the ABCG2 inhibitor fumitremorgin C, but no

reduction of SP proportion was seen. It is known that ABCG2 transporters and phosphorylated p-glycoprotein (P-gp) are highly expressed in normal and cancerous stem cells [5]. Western blot assay for ABCG2 and P-gp expression in the lymphoma cells revealed higher expression levels of membrane transporter proteins such as ABCG2 and P-gp, required for the Hoechst efflux. Elevated expression levels of ABCG2 proteins in the adenoma samples may be an explanation for our results. However, no protein or gene expression analysis of the sorted samples was performed.

Besides the 3 adenoma samples, 2 healthy pituitary samples were not affected by verapamil. These findings could be explained by an actual cell density in the samples of $>10^6$ cells/ml during Hoechst staining. Percentage life cells calculated on the cell counter before Hoechst staining, differed from percentage life cells measured during FACS analysis. Percentage life cells prior to Hoechst staining in the 2 healthy pituitary samples was calculated to be 14 and 31%. During FACS analysis, percentage life cells was much higher and calculated to be 73 and 79.5%, respectively. Mo *et al* [12] calculated the SP fraction of PI-negative cells in samples with a different cell density but the same Hoechst concentration, and reported an increased SP fraction with increased cell density. Since the Hoechst concentration was not adjusted, relatively less Hoechst was available per cell. Hoechst is a cell-permeable DNA-specific dye and SP cells are detected due to their ability to efflux Hoechst. Due to an increase in percentage life cells in the 2 healthy pituitary samples, cell density increased and relatively less Hoechst and verapamil was available. Because there was less verapamil available, possibly not all ABCG2 transporters were blocked. Besides, there was not enough Hoechst available to stain all the DNA of all the cells and thus, cells were low in red emission and appeared in the dot plot at the same location as the SP cells. Consequently, measured SP fraction increased and no inhibition in the control samples was seen.

The fraction SP cells defined in the canine healthy pituitaries, as well as in the canine pituitary adenomas, differed among the samples. Fraction SP cells measured on a FACSAria II (SORP) in healthy pituitaries ranged from 0.1 to 2.9% (mean 0.70±0.96%), and in pituitary adenomas from 0.1 to 4.1% (mean 1.01±1.36%). The high variance in SP fraction may be explained by variations in cell density, viable cell proportion, staining volume and the use of clinical samples. Mo *et al* [12] reported a drop in SP fraction with an increase of PI-negative cells. In the present study there was a high variance in cell viability among the samples (2-44%) and percentage life cells was significantly correlated with the proportion SP cells (P=0.040). Samples with better cell viability had smaller SP proportions, which is similar with the results presented in the study of Mo *et al*. In our study, cell density was adjusted to 10^6 cells/ml in all samples, as described previously [7,8]. However, a difference was found between percentage life cells prior to Hoechst staining and during FACS analysis, which may have influenced actual cell density. Besides, staining volume differed among the samples due to the high variance in total life cell count. Mo *et al* reported a decreased SP proportion with an increased staining volume. Control samples incubated with Hoechst and verapamil were stained in less volume (0.1–0.5 ml) compared with the samples stained with only Hoechst (0.5-4.0 ml). Though, the control sample and sorting sample were compared and used to set the right sorting gate for SP isolation.

In the present study, a modified protocol of Denef *et al* [9] was used for cell digestion, as described previously [8]. It was not possible to give a clear explanation for the high variance in total life cell count and cell survival among the healthy pituitary and adenoma samples. To investigate whether preservation or the kind of medium used in the digestion protocol effects cell survival, a clear

experimental design must be used, with changing one parameter at once and testing in only one sample type.

In all healthy pituitary and pituitary adenoma samples, a SP could be identified. However, SP appearance differed among the samples. The use of patient material and with that, high variation in total life cell count, percentage life cells and possibly DNA content, resulted in variations of staining volume and cell density, and with that, SP proportion. To sort the correct SP proportion and to compare SP proportions among the different samples, less variation in staining conditions is preferred.

5.5 Cited references

[1] Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med 1996;183:1797-806.

[2] Asakura A, Rudnicki MA. Side population cells from diverse adult tissues are capable of in vitro hematopoietic differentiation. Exp Hematol 2002;30:1339-45.

[3] Brown MD, Gilmore PE, Hart CA, Samuel JD, Ramani VAC, George NJ et al. Characterization of benign and malignant prostate epithelial Hoechst 33342 side populations. Prostate 2007;67:1384-96.

[4] Wu C, Wei, Q, Utomo, V, Nadesan P, Whetstone H, Kandel R, Wunder JS et al. Side population cells isolated from mesenchymal neoplasms have tumor initiating potential. Cancer Res 2007;67:8216-22.

[5] Kim M, D'Costa S, Suter S, Kim Y. Evaluation of a side population of canine lymphoma cells using Hoechst 33342 dye. J Vet Sci 2013;14:481-6.

[6] Chen J, Hersmus N, Van Duppen V, Caesens P, Denef C, Vankelecom H. The adult pituitary contains a cell population displaying stem/progenitor cell and early embryonic characteristics. Endocrinology 2005;146:3985-98.

[7] Chen J, Gremeaux L, Fu Q, Liekens D, Van Laere S, Vankelecom H. Pituitary progenitor cells tracked down by side population dissection. Stem Cells 2009;27:1182-95.

[8] van Rijn SJ, Gremeaux L, Riemers FM, Brinkhof B, Vankelecom H, Penning LC et al. Identification and characterisation of side population cells in the canine pituitary gland. Veterinary Journal 2012;192:476-82.

[9] Denef C, Maertens P, Allaerts W, Mignon A, Robberecht W, Swennen L et al. Cell-to-cell communication in peptide target cells of anterior pituitary. Methods in enzymology 1989;168:47-71.

[10] Langouche L, Hersmus N, Papageorgiou A, Vankelecom H, Denef C. Melanocortin peptides stimulate prolactin gene expression and prolactin accumulation in rat pituitary aggregate cell cultures. Journal of neuroendocrinology 2004;16:695-703.

[11] Simpson C, Pearce DJ, Bonnet D, Davies D. Out of the blue: A comparison of Hoechst side population (SP) analysis of murine bone marrow using 325, 363 and 407 nm excitation sources. J Immunol Methods 2006;310:171-81.

[12] Mo SL, Li J, Loh YS, Brown RD, Smith AL, Chen Y et al. Factors influencing the abundance of the side population in a human myeloma cell line. Bone Marrow Res 2011;Epub.

6. RNA quality after FACS analysis of canine healthy pituitary and pituitary adenoma cells

6.1 Introduction

RNA degradation is a problem in all experimental settings, and for clinical samples in particular. It has been reported that an increased post-mortem interval (PMI) decreases RNA quality in human brain tissue and with that, alterations in gene expression [1]. Koppelkamm *et al* [2] analyzed the effect of RNA degradation on quantitative gene expression in post-mortem brain, skeletal and cardiac muscle tissue. Depending on transcript and tissue type, a difference in expression of up to two threshold cycles was found. Others report only a small number of genes to be affected by RNA degradation, particularly short RNA molecules or those with probe positions near the 5' end [3]. Romero *et al* [4] described a protocol to correct quantitative gene expression for RNA degradation, which makes it possible to use highly degraded samples. Even though correction makes the detection of large differences in gene expression still possible, small differences may be missed or misinterpreted [3]. Therefore, high RNA quality is preferred, to compare quantitative gene expression among the sorted samples.

RNA degradation is caused by RNases and contamination of samples with RNases will lead to decreased RNA quality. RNases, or ribonucleases, are enzymes capable to cleave nucleotides of RNA strands. Endonucleases cut RNA internally, whereas exonucleases degrade RNA at the 5' or 3' end [5]. In addition to cellular RNases that are released during cell damage, there are several RNases that are present in the environment, for example in the FACS machine. To sort cells by means of FACS, cells are forced into a thin stream. The cells flow with high speed through a laser beam and the nozzle separates the stream into single droplets. The cells are charged and deflected in an electrostatic field, so that they fall into the right collection tubes [6]. Mollet *et al* reported that exposure to hydrodynamic forces only caused a minor increase in apoptosis in Chinese hamster ovary (CHO) cells [7]. However, another paper published by the same research group reports a significant cell damage in CHO cells and THP1 cells, a human acute monocytic leukemia cell line [6]. Besides these reports, little research is done about cell damage and FACS analysis. However, apoptosis and cell damage due to the staining procedure prior, or hydrodynamic forces during FACS analysis, may decrease RNA quality.

The RNA quality in samples can be measured spectrophotometrically or by means of electrophorese. The ratio of the absorbance at 260 nm and 280 nm (260/280) is used to assess the purity of nucleic acids on the Nanodrop. A ratio of 2.0 is generally accepted as 'pure' RNA. A lower ratio indicates the presence of proteins, phenol or other contaminants that absorb to the 280 nm (manufacturers guidelines). The 260/280 ratio only provides information about protein or phenol contamination, but does not give full information about RNA integrity [8]. Using the Agilent Bioanalyzer, information about RNA integrity is provided. The sample is separated by electrophoresis in order to molecular weight of the RNA molecules. RNA strands are detected by laser-induced fluorescence detection and the amount of fluorescence correlates with the amount of RNA of a given size [9]. Quality is determined on the 26s/28s ribosomal ratio and with an algorithm RNA integrity number (RIN) value is calculated, with '1' for highly degraded RNA and '10' for highly intact RNA [8,9].

To compare quantitative gene expression in the sorted SP and MP cells from healthy pituitary and pituitary adenoma samples, high quality RNA is needed. The aim of this study was to investigate the best collection method in order to obtain high quality RNA from sorted canine healthy pituitary and pituitary adenoma cells.

6.2 Materials & Methods

6.2.1 Experimental design

First, bone marrow cells were incubated with the dye Hoechst 33342 and sorted using FACS. To investigate which collection method gives the best RNA quality and quantity, different collection methods were tested. Second, FACS analysis was performed on both pituitary gland and adenoma samples, using two different FACS machines. Cells were sorted and collected in RNA *Later®,* RLT-buffer (RNeasy microkit, Qiagen), or in empty tubes. RNA quantity and quality were measured on both the Nanodrop ND-1000 (Isofen Life Sciences, IJsselstein, the Netherlands), or Agilent Bioanalyzer 2100 (Agilent technologies). RNA yields and RIN values were related to tissue type, SP/MP, fresh or frozen samples, FACS instrument, amount of sorted cells and percentage life cells in the samples. Quantitative gene expression analysis was performed on a subset of the samples and threshold cycles (Ct) were related to RIN value.

6.2.2 Sample collection

Bone marrow cells were collected from a healthy, 3 year old female dog, euthanized in a non-related experiment. One hind limb was washed twice with Hibiscrub® to remove loose hairs, followed by two washes with Hibisol®. Tissue and muscles were removed with a surgical blade nr. 10 and the femur was dissected. The bone was sawed using an electrical saw on both sides in the metaphysis and was kept in prewarmed α-MEM (GIBCO, Invitrogen) enriched with heparin (LEO Pharma, Ballerup, Denmark), 2% FCS and 1% penicillin streptomycin until further processing. Healthy pituitary and pituitary adenoma samples were collected as described in chapter 5.2.2.

6.2.2 Cell dispersion and Hoechst staining

To collect the bone marrow cells, the bone was excavated using a Needle holder (Mayo-Hegar) and flushed with prewarmed α-MEM enriched with heparin, 2% FCS and 1% penicillin streptomycin. After red blood cell (RBC)-lysis, the cell suspension was washed three times with HBSS+2%FCS, filtered and resuspended in prewarmed DMEM glutamax enriched with 2% FCS, 10mM Hepes and 0.4% insulin in a concentration of $1x10^6$ cells/ml. Hoechst staining was performed as described in chapter 5.2.4, using 5 µg/ml Hoechst 33342 assessed by Goodell *et al* [10]. To the control samples, 9.1 µl/ml Verapamil was added.

Cell dispersion and Hoechst staining of the healthy pituitary gland and pituitary adenoma was performed according to the protocols described in chapter 5.2.3 and 5.2.4. To investigate whether the type of medium used in the staining procedure affects the RNA quality, different kinds of media were used (Table 6.1). Media 1A or 2A were used during Hoechst staining and after incubation, the cells were resuspended in the so-called sorting solution, media 1B or 2B. The composition of these media is described in the previous chapter (Chapter 5.2.3 and 5.2.4).

6.2.3 FACS analysis

FACS was performed at the Hubrecht Institute, Utrecht, on a FACSAria II (SORP) and at the department of immunology, faculty of Veterinary Medicine, Utrecht, on a BD influx. Both FACS instruments sort with a speed ranging from 200-1000 and 600-2000 events/sec, respectively. Specifications of the two FACS machines are listed in table 5.3 of the previous chapter.

6.2.4 Collection of the sorted cells

Cells were collected by different collection methods, as shown in table 6.1. For collection of the bone marrow cells, collection methods A to H were used, using for every sample medium 2A and 2B. For collection of the pituitary gland cells, collection methods C, D, E or G were used. In collection method A, cells were not sorted, but stained for 90 minutes with Hoechst, resuspended in medium 2A and kept for 3 hours in a flask at 37°C, previously to RNA isolation. In collection methods B and C cells were collected in 350 µl RLT buffer with 4 µl β-Mercaptoethanol (β-ME)(Sigma-Aldrich) and kept on ice or snap frozen, respectively. When collected in RLT buffer, β-ME was added immediately after sorting to avoid toxic fumes. With collection method D, β-ME was added after the cells were transported back to our own laboratory, approximately 1 hour after sorting. Cells were collected in tubes containing RNA *Later®*, with collection methods E and F, and in empty tubes using collection methods G and H. The time and speed of centrifuging differed among the collection methods(table 6.1). Compared with the bone marrow cells, altered medium was used to collect pituitary gland cells using collection method C and E, media 1A/1B instead of 2A/2B. Previously to sorting, all tubes were kept on ice.

6.2.5 RNA isolation

RNA was isolated from the SP and MP of healthy pituitary and pituitary adenoma samples, as well as from sorted bone marrow cells, using the RNeasy micro kit (Qiagen), according to the manufactures guidelines. After sorting, samples were kept on ice or snap frozen, and transported back to our lab prior to RNA isolation. Samples collected in RNA *Later®* were stored at -70°C prior to RNA isolation. RNA quality and quantity were measured spectrophotometrically using a Nanodrop ND-1000, and with an Agilent Bioanalyzer 2100. RNA quality measured with an Agilent Bioanalyzer 2100 was given in RIN.

Collection method	Media	Collection method	Centrifuged	Nr. of samples	
				Bone marrow	Pituitary gland
A	2A/2B	Hoechst staining protocol, not sorted by FACS	-	2	0
B	2A/2B	350 µl RLT-buffer + β-ME; kept on ice	-	2	15
C	1A/1B*[1] 2A/2B	350 µl RLT-buffer + β-ME; snap frozen	-	2	10
D	2A/2B	350 µl RLT-buffer, back in our own laboratory β-ME is added; Kept on ice	-	2	2
E	1A/1B*[1] 2A/2B	Tube containing 1 ml RNA *Later®;* Kept on ice	5 min; 3000 rpm	2	24
F	2A/2B	Tube containing 3 ml RNA *Later®*; Kept on ice	30 min; 4000 rpm	2	0
G_1	2A/2B	Empty, ice cold 1 ml tube; Kept on ice	5 min; 3000 rpm	2	2
G_2	2A/2B	Empty, ice cold 1 ml tube;	10 min; 3000 rpm	0	2
G_3	2A/2B	Empty, ice cold 1 ml tube; Kept on ice	10 min; 2000 rpm	0	1
H	2A/2B	Empty, ice cold 15 ml tube; Kept on ice	10 min; 2000rpm	2	0

Table 6.1: Different collection methods were used to collect bone marrow cells and pituitary gland cells after cell sorting. Not every collection method was used for both tissue types. Healthy pituitary samples collected in RNA *Later®* were stored at -70°C prior to RNA isolation. Medium 1A consisted of 1 liter DMEM 1:1/F12 containing 5 mg transferrin, 5 mg Insulin, 1% penicillin streptomycin, 1 mg catalase and 1 g NaHCO3. Medium 1B was HBSS, containing 2% FCS, 2µg/ml propidium iodide (PI), 0.4% DNase and 0.025% EDTA. Medium 2A consisted of DMEM Glutamax, 2% FCS+ 10 mM Hepes + 0.4% insulin and medium 2B was HBSS enriched with 2% FCS and 2µg/ml PI. When collecting in RLT buffer, β-ME was added directly after sorting, except in collection method D. RNA isolation was performed directly after FACS using a RNeasy microkit (Qiagen). Samples collected in RNA *Later®* were stored at -70°C previously to RNA isolation. *[1] For the pituitary gland samples, medium 1A/1B was used, and for the bone marrow cells, media 2A/2B. Abbreviations: β –ME, β-Mercaptoethanol; rpm, rounds per minute.

6.2.6 Quantitative PCR

Quantitative gene expression analysis was performed on a subset of the sorted samples. cDNA was synthesized with variable input using the iScript™ cDNA Synthesis Kit (Bio-rad, Veenendaal, the Netherlands) according to manufacturer's instructions. The RT-qPCR reaction was performed in duplicate on a BioRad I-Cycler (Bio-Rad). For each qPCR sample, a total volume of 10 µL was used, containing 5.0 µL IQ SYBR green SuperMix (Bio-Rad), 4.0 µL of 50-fold diluted cDNA, and 100 µM of both forward and reverse primers. Gene expression of the reference genes *hmbs, tbp* and *ywhaz* was analyzed, previously identified as the most stable reference genes for pituitary and adenoma samples [11]. Details of the primers used in this study are depicted in Table 6.2.

Gene	NCBI Ref. seq.	F/R	Sequence	Exon	Amplicon size	T_a (°C)
hmbs	XM_546491	F R	5'-TCACCATCGGAGCATCT-'3 5'-GTTCCCACCACGCTCTTCT-'3	6 7	112	61
tbp	XM_849432	F R	5'-CTATTTCTTGGTGTGCATGAGG-'3 5'-CCTCGGCATTCAGTCTTTTC-'3	5 5	96	58
ywhaz	XM_533072	F R	5'-CGAAGTTGCTGCTGGTGA -'3 5'-TTGCATTTCCTTTTTGCTGA-'3	2 3	94	58

Table 6.2: Nucleotide sequences, exon, amplicon size and annealing temperature (T_a) of the primers used for RT-qPCR. Hmbs,hydroxymethylbilane synthase; tbp, tata box binding protein; ywhaz, ,tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide. Abbreviations: F, forward primer; R, reverse primer;

6.2.7 Statistical analysis

Statistical analysis was performed in IBM SPSS (22). Measurements of RNA quality are more accurate on the Agilent Bioanalyzer 2100 [8], and thus, these values were used for the analysis. To test for normal distribution, a Shapiro-Wilk test was used and a Spearman's Rho test was performed to test for correlations between the different parameters. To compare quantity measured in paired samples on the Nanodrop ND-1000 and Agilent Bionalayzer 2100, a Wilcoxon signed-rank test was performed. The Mann-Whitney U test was used to investigate whether there was a significant difference between RNA yields in pituitary and adenoma samples, fresh or frozen samples, the different FACS instruments, media 1A/1B or 2A/2B, and SP or MP. To compare RNA quantity and quality among the different collection methods, a Kruskal-Wallis test was performed. Significance was set at $P < 0.05$.

6.3 Results

6.3.1 FACS analysis

All bone marrow samples were sorted using a FACSAria II (SORP). As described in chapter 5.3.2, all canine pituitary adenomas (17) and 14 healthy canine pituitaries were sorted using a FACSAria II (SORP), and all feline pituitary adenomas (3) as well as 5 healthy pituitaries were sorted using a BD Influx (table 6.3). The samples were sorted in a SP and a MP and the amount of sorted cells ranged from 64 to 140,000 (mean 29,870 ± 97,525 SD) for the SP and 503 to 150,000 (mean 17,019 ± 29,546 SD) for the MP with a median of 5053 and 5660, respectively.

	FACSAria II (SORP)		BD Influx	
Sample type	Healthy pituitary	Canine pituitary adenoma	Healthy pituitary	Feline pituitary adenoma
Analyzed samples	14	17	5	3
Sorted samples	14 SP 15* MP	17 SP 17 MP	5 SP 5 MP	3 SP 3 MP
RNA isolation	12 SP 12 MP	6 SP 6 MP	5 SP 5 MP	3 SP 3 MP
qPCR	3 SP 4 MP	-	5 SP 5 MP	3 SP 3 MP

Table 6.3: The amount of samples analyzed by FACS and sorted in SP and MP. A subset of the sorted samples was used for RNA isolation and qPCR. * of one sample, one SP and two MP samples were sorted. qPCR, quantitative PCR.

Total life cell count of the samples (depicted in table 5.1 of the previous chapter) and the amount of sorted SP and MP cells (6.5, supplementary), is significantly correlated (SP, *P* = 0.006, correlation coefficient (CC) = 0.463; MP, *P* <0.001, CC 0.656). Besides, the amount of sorted SP cells

was significantly correlated with the percentage life cells in the sample prior to (P = 0.011, CC = 0.397).

6.3.2 RNA quantity and quality in bone marrow samples

Eight different collection methods were used to collect bone marrow cells and for every collection method, two samples with each 30.000 cells were sorted. Quantity and quality measured on the Nanodrop ND-1000 and Agilent Bioanalyzer 2100, are listed in table 6.4. On the Agilent Bioanalyzer 2100, RNA integrity of >6 RIN was measured in samples collected according to collection methods A, B, C, F, G and H. RNA yields ranged from 92 to 663 pg/µl and the lowest RNA yield was measured in the sample collected in RNA *Later®* and centrifuged (30 min, 4000 rpm). The highest RNA yield was measured in the sample collected in 350 µl RLT buffer with β-ME. No statistical analysis was performed to test for differences in RNA yields in the bone marrow samples.

6.3.3 RNA quantity in pituitary samples

RNA was isolated of a subset of the sorted pituitary gland samples (table 6.3). RNA was isolated of the SP and MP of 12 healthy pituitary samples as well as from the SP and MP of 6 canine pituitary adenoma samples, sorted with the FACSAria II (SORP). Of all samples sorted with the BD Influx, RNA was isolated. RNA quantity of all samples was measured on the Nanodrop ND-1000 and was given in ng/µl. RNA quantity ranged from 2.4 to 39.4 (mean 7.8 ± 6.5 SD), with a median of 5.8. Of 35 samples, RNA quantity was also measured on the Agilent Bioanalyzer 2100. Quantity was given in pg/µl and ranged from 0 to 7230 (mean 532.4 ± 1398.9 SD) with a median of 71.0. There was no significant difference in RNA quantity (calculated to pg/µl) between the samples measured with the Nanadrop ND-1000 and Agilent Bioanalyzer 2100 (P = 0.318). In table 6.5 (supplementary) RNA quantity of all the healthy pituitary and pituitary adenoma samples is listed.

Quantity measured on the Agilent Bioanalyzer 2100 was used to compare the different protocols. The amount of sorted cells and quantity was significantly correlated (P < 0.01, CC 0.580). Since a correlation was found and different amounts of cells were sorted, RNA quantity was calculated for 30.000 cells and this corrected quantity was used to perform statistical analysis (table 6.5, supplementary). There was no statistical significant difference in RNA quantity and collection method (P = 0.608), pituitary adenoma or healthy pituitary (P = 0.876), media 1A/1B or 2A/2B (P = 0.972), fresh or frozen sample (P = 0.563) FACS instrument (P = 0.563) and SP or MP (P = 0.608). In addition, percentage life cells prior to Hoechst staining and RNA yields were not correlated (P = 0.412). In figure 6.1, boxplots of RNA quantity, grouped for the different protocols, are represented.

In figure 6.2, a boxplot is depicted in where the RNA quality and quantity of two groups of samples are compared. Samples depicted in blue were all freshly processed with media 2A/2B, sorted on a BD Influx and collected in RLT buffer + β-ME. Samples depicted in green were all frozen, processed with media 1A/1B, sorted on the FACSAria II (SORP) and collected in RNA *Later®*. The groups contain both healthy pituitary and pituitary adenoma samples, as well as SP and MP cells. There was no significant difference between both groups in RIN value (P = 0.625) or RNA quantity (P = 0.705).

RNA quality and quantity in sorted bone marrow samples						
			Nanodrop		Bioanalyzer	
	Protocol	Sample nr	Quantity (ng/µl)	Quality (260/280)	Quantity (pg/µl)	Quality (RIN)
A	Hoechst staining protocol; Not sorted by FACS	1	31.1	1.82		
		2	7.9	1.85	653	7.5
B	RLT-buffer + β-ME; kept on ice	1	6.5	1.86		
		2	7.6	1.67	428	8.6
C	RLT-buffer + β-ME; Snap frozen	1	6.2	1.62		
		2	4.5	1.85	538	7
D	RLT-buffer; back in our own laboratory β-ME is added; Kept on ice	1	4.8	1.25		
		2	7.6	1.56	634	N/A
E	Tube containing 1 ml RNA *Later®;* Kept on ice; 5 min/3000 rpm	1	10.4	1.34		
		2	7.2	1.56	303	N/A
F	Tube containing 3 ml RNA *Later®*; Kept on ice; 30 min 4000 rpm	1	6.0	1.48		
		2	4.9	1.54	92	7.1
G	Empty, ice cold 1 ml tube; Kept on ice; 5 min 3000 rpm	1	5.5	1.11		
		2	4.3	1.52	663	7.2
H	Empty, ice cold 15 ml tube; Kept on ice; 10 min 2000 rpm	1	6,3	1.37		
		2	6.9	1.44	658	6.9

Table 6.4: RNA quality and quantity was measured in sorted bone marrow samples on the Nanodrop ND-1000 (Isofen Life Sciences) and Agilent Bioanalyzer 2100 (Agilent technologies). Per sample 30.000 cells were sorted. High RIN values (>7) were seen in protocol A, B, C, F and G. FACS analysis was performed on a FACSaria II (SORP) at the Hubrecht Institute, Utrecht.

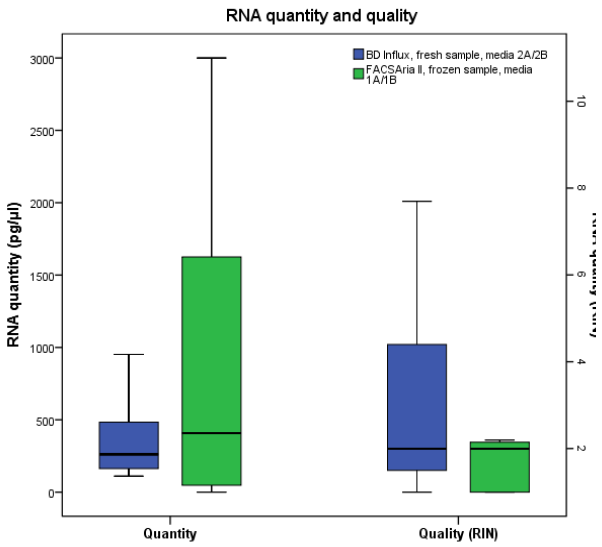


Figure 6.2: RNA quantity (pg/µl) and quality (RIN) of two groups of samples. Groups contain both healthy pituitary and pituitary adenomas, and SP and MP samples. There are no overlapping parameters of the protocols used. No significant difference was found between both groups in RIN value (*P* = 0.625) or RNA quantity (*P* = 0.705). Blue (n=8), green (n=16).

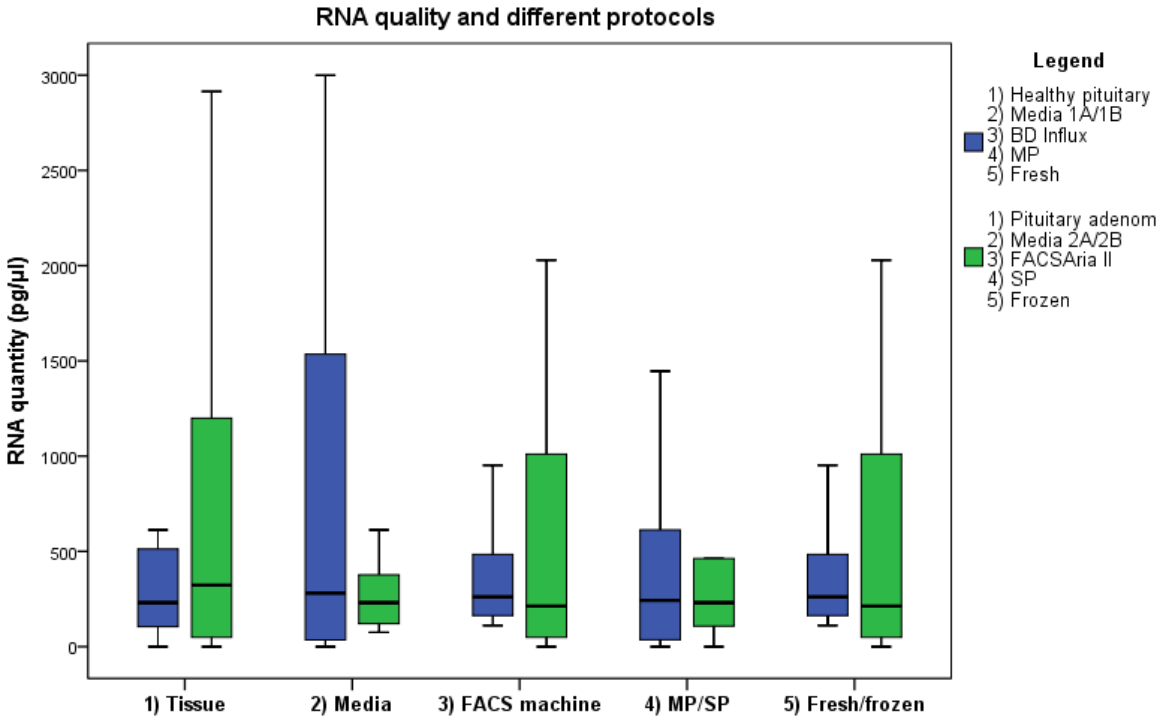


Figure 6.1: RNA quantity measured in samples that were prepared and sorted according to different protocols. RNA was measured on the Agilent Bioanalyzer in 35 samples, and RNA quantity is given in pg/µl. Quantity was calculated for 30.000 cells. RNA quantity was compared in all samples and every boxplot displays a difference in one parameter. 1) tissue: healthy pituitary (n=23)(1430.9±4526.8) vs pituitary adenoma (n=12)(690.4±898.6); 2) media: media 1A/1B (n=24)(1573.8±4429.6) vs media 2A/2B (n=12)(311.3±265.7); 3) FACS instrument: BD Influx (n=7)(373.8±306.9) vs FACSAria II (n=28)(1377±4117,8); 4) MP/SP: MP (n=18)(514,4±653,7) vs SP (n=17)(1878,6±5247,0); 5) fresh/frozen: fresh (n=7)(373.8±306.9) vs frozen (n=28)(1377.0±4117.8). No significant difference was found between RNA quantity and one of the parameters. The composition of media 1A/1B and 2A/2B is depicted in chapter 5.2.3 and 5.2.4. Abbreviations: MP, main population; SP, side population.

6.3.4 RNA quality in pituitary samples

RNA quality determined on the Nanodrop ND-1000 was given in a 260/280 ratio and ranged from 1.08 to 4.63 (mean 1,69±0,72 SD), with a median of 1.52. RIN values were given for quality measured on the Agilent Bioanalyzer 2100 and ranged from 1 to 8.1 (mean 2.65±2.42 SD) with a median of 1.1. Quality measured with both methods in paired samples was not correlated (P = 0.320). The RIN values of samples collected by different methods, are grouped in figure 6.3. RIN values of >6 were found in samples collected by collection method B, E and G_1. There was no significant difference in RIN values between collection method B, C and E (P = 0.600). Besides, no significant difference in RIN values was found between pituitary adenoma or healthy pituitary (P = 0.165), media 1A/1B or 2A/2B (P = 0.631), fresh or frozen sample (P = 0.564), FACS instrument (P = 0.564) and SP or MP (P = 0.468). In table 6.5 (supplementary) quality of all the healthy pituitary and pituitary adenoma samples is listed.

Collection method		N/A	RIN 1	RIN 2	RIN 3	RIN 4	RIN 5	RIN 6	RIN 7	RIN 8	RIN 9	RIN 10
B	*(n=7)*	2	2	1	0	0	1	0	1	0	0	0
C	*(n=8)*	5	3	0	0	0	0	0	0	0	0	0
D	*(n=2)*	0	2	0	0	0	0	0	0	0	0	0
E	*(n=16)*	3	8	0	1	0	0	3	0	1	0	0
G1	*(n=2)*	0	1	0	0	0	0	0	1	0	0	0

Figure 6.3: Sorted healthy pituitary and feline and canine pituitary adenoma samples were grouped according to collection method (protocol B, C, D, E, G1) and quality (RIN). RIN values were round off downwards (if RIN < 0.5) or upwards (if RIN ≥ 0.5).

6.3.5 Quantitative PCR

Quantitative RT-PCR was performed on 13 sorted SP and MP samples, originating of 6 canine healthy pituitary samples (table 6.3). The healthy pituitary samples were sorted on two different FACS machines, the FACSAria II or BD Influx. All samples sorted on the FACSAria II (SORP) were stored in liquid nitrogen previously to cell digestion. The samples sorted on the BD Influx were freshly processed. Ct values of three reference genes, *hmbs*, *tbp* and *ywhaz*, were determined. Mean Ct value of the samples sorted on the FACSAria II (SORP) was 32.99 ± 2.14 and mean Ct value of the samples sorted on the BD Influx was 30.5 ± 2.36. There was no statistical difference between mean Ct values (P = 0.077) of samples sorted on the different FACS machines. Gene expression analysis was performed with variable input, with a median input of 756 pg (793.33 ± 483.38 SD). Ct values were not correlated with the input (P = 0.412) or RIN values of the samples (P = 0.406).

The three reference genes were tested in duplo, resulting in 6 measurements per sample. In figure 6.4, percentage of the samples that did show gene expression is depicted. Except for one sample that did not express *tbp* in duplo, gene expression could be measured in all samples sorted on the BD Influx. Samples sorted on the FACSAria II (SORP) did not show consistent gene expression, and in only one out of the 7 samples expression in all genes (in duplo) could be measured.

BD Influx	100	100	83	100	100	100
FACSAria II	43	29	29	29	71	100
	hmbs (1)	hmbs (2)	tbp (1)	tbp (2)	ywhaz (1)	ywhaz (2

figure 6.4: Percentage (%) of samples that a showed gene expression. Expression for *hmbs, tbp* and *ywhaz* was measured in duplo. All samples consisted of sorted SP or MP cells from healthy canine pituitary samples. BD Influx (n=6), FACSAria II (n=7).

6.4 Discussion

In many tissues, a SP is successfully sorted by means of FACS analysis. To analyze and compare the gene expression profiles of sorted samples, high RNA quality is preferred. The aim of the present study was to investigate the best collection method for cells after sorting, in order to obtain high quality RNA from canine healthy pituitary and pituitary adenoma cells. Cells were sorted and

collected directly in the RNA extraction buffer, or collected in empty tubes or tubes with RNA *Later*® and centrifuged prior to RNA isolation.

RNA *Later*® is a nontoxic tissue storage reagent which inactivates RNases and permeates tissue to stabilize and protect the integrity of RNA (manufactures guidelines). An advantage of collecting the cells in RNA *Later*® instead of empty tubes is the stabilization of RNA, an disadvantage is the higher viscosity of the liquid and thus, prolonged centrifuge times and loss of cells and RNA. In a study of Nishimoto *et al* [12], a protocol was described which results in a 70-80% recovery of cells and high RNA quality. Samples were collected in 10 ml tubes with RNA *Later*®, resulting in a 10-20% dilution, and centrifuged for 30 minutes at 2500 *g*. In the present study, different time and speed settings were investigated. Both pituitary and bone marrow cells were collected in 1 ml RNA *Later*®, centrifuged (5 min; 3000 rpm) and RNA yields ranged from 0 to 7230 pg/µl per 14,000 and 150,000 cells in pituitary samples and 303 pg/µl per 30.000 cells in bone marrow samples. Low RNA yields when collecting in RNA *Later*® could be due to the high viscosity of the liquid and longer centrifuge times may be indicated. However, centrifuging for 30 minutes at 4000 rpm gave a poor yield (92 pg/µl) as well. A possible explanation is damage to the cells due to the high speed and extended centrifuge time, and thus loss of RNA.

Samples of cells collected in empty tubes and centrifuged (5 min; 3000 rpm), gave moderate to good RIN values in the bone marrow, and poor to moderate RIN values in the pituitary samples. Of 30.000 sorted bone marrow cells, 663 pg/µl RNA could be isolated. In pituitary cells RNA yields were 49 and 63 pg/µl of 3677 and 25,000 sorted cells respectively. As for collection in RNA *Later*®, extended centrifuge time and lower speed was tested. However, for these protocols there are no data of values measured on the Agilent Bioanalyzer 2100, which is more accurate for small amounts of RNA.

RNA degradation is caused by RNases that are released during cell damage, or that are present in the environment. The samples sorted in this study could have been contaminated with RNases from the environment. During FACS analysis, no RNase-free sorting solution, but PBS was used, and no RNA protector was added. Besides, cell death after FACS, and with that the release of RNases from the cytoplasm, may have led to RNA degradation. In a study of Mollet *et al* [6] cell death after FACS analysis was investigated. THP1 and CHO cells were sorted with different flow rates and nozzles, leading to different hydrodynamic forces. A higher flow through the FACS resulted in significant more cell damage. Besides, a rapid increase of cell damage was found when the hydrodynamic force slightly increased. Cells were sorted with a pressure of 19 PSI and speed of 200-1000 events per second with the FACSAria II (SORP), and with a pressure of 25 PSI and a speed of 600-2000 events per second with the BD Infux. Increased cell damage, and with that lower RIN values, were expected in the samples sorted with the BD Influx. RIN values were not determined of all samples sorted on the BD Influx, which makes it hard to compare both instruments. However, samples sorted with the BD Influx instrument show a more stable expression of the tested reference genes.

To avoid RNA degradation, cells could be collected directly in lysis buffer. In collection method B, C and D cells were collected in RLT buffer, the first step of the RNA isolation protocol using the RNeasy micro kit. RLT buffer is a lysis buffer with a high concentration of guanidine isothiocycanate, which supports the binding of RNA to the silica membrane of the RNA extraction column (manufactures guidelines). β-ME irreversibly denatures RNases by reducing disulfide bonds and destroying the

native conformation required for enzyme functionality. To avoid toxic fumes, β-ME was added directly after the cells were sorted. In protocol D, β-ME is added back in our own lab, approximately 1 hour after sorting. In both bone marrow and pituitary samples, RNA quality was very poor or not applicable when β-ME was not added directly. This indicates the important role of β-ME in inactivating RNases and thus, protecting RNA.

Higher RIN values were measured in all bone marrow samples compared with pituitary samples collected by the same protocol. A difference between the pituitary and bone marrow samples is the cryopreservation of the tissues. Bone marrow cells were collected from a fresh cadaver dog and processed immediately, while pituitary samples were collected in Leibowitz+ 10%FCS+ 10%DMSO, frozen, stored for 1 month up to 4 years and thawed on the day of FACS analysis. Some healthy pituitary and all feline pituitary adenoma samples were immediately digested and kept overnight at 37°C prior to sorting Less cell viability was seen in the fresh samples (chapter 5.3.1) and there was no statistical difference in RNA yields or quality between fresh and frozen samples. In a study of Eikmans *et al* [13], the effect of cryopreserving of human blood cells on RNA integrity was investigated and the same results were found. RNA isolation was performed directly after sample collection, after storage for one day at -80°C or after storage for 3 days at -180°C in culture medium (RPMI1640) with 20% FCS and 10% DMSO. There was no significant difference in RNA integrity of RNA quantity extracted from freshly processed cells, or from thawed cells after freezing in culture medium for 1 or 3 days. Apart from cryopreservation, post-mortem sampling and a prolonged PMI might be an explanation for the low RNA yields and integrity in the pituitary samples collected in empty tubes, compared with bone marrow samples. Some tissues are more vulnerable for RNA degradation than others, as Koppelkamm *et al* investigated [2]. RNA quality in human post-mortem samples was examined and significantly lower integrities were found in brain, compared to skeletal and cardiac muscle. These results indicate that RNA integrity depends on tissue type rather than cryopreservation. However, no research in done on post-mortem pituitary samples, and no information is available about the PMI of all healthy pituitary samples used in our study.

In figure 6.1 box plots of RNA yields compared to different parameters were depicted. It is hard to compare the different parameters among each other, since changes in protocol were not consistent. For example, all fresh samples were sorted using the BD Influx, and all frozen samples with the FACSAria II (SORP). Media 1A/1B was used in all samples, except 4, sorted on the FACSAria II (SORP), whereas media 2A/2B was used in all samples sorted with the BD Influx. All canine pituitary adenomas were sorted on the FACSAria II (SORP), and all feline pituitary adenomas were sorted on the BD Influx. Different amounts of samples were collected in order to test the best collection method. Some collection methods were only used for two samples, which did not give enough data to statistically compare all collection methods. Because of the overlap in parameters, it is difficult to conclude which protocol gives the highest RNA yields and best quantity. However, collecting in RLT buffer + β-ME was preferred, since the highest RIN value was measured in the bone marrow sample collected by this method (RIN 8.1). When collecting in RLT buffer, centrifugation is not needed, with likely less cell loss. Besides, β-ME inactivates RNases and thus, less RNA degradation is expected.

To investigate the influence of protocol, FACS machine and RNA integrity on quantitative gene expression, qPCR was performed on a subset of the samples. Ct values of fresh samples sorted on a BD Influx were compared with Ct values of frozen samples sorted on a FACSAria II (SORP). Mean Ct differed more than two threshold cycles, but the difference was not significant. Significance was

tested with only Ct values of reference genes (*hmbs, tbp, ywhaz*) that were expressed in the samples, but not all genes were expressed in all samples (figure 6.4). Of the samples sorted on the BD Influx, all samples (except one) expressed all genes in duplo, whereas samples sorted on the FACSAria II (SORP) did not show stable expression. All samples sorted on the BD Influx were processed fresh, and all samples sorted on a FACSAria II (SORP) were stored at -190˚C before use. It is not possible to conclude if the difference in expression profile is due to the type of FACS instrument or due to the preservation of the samples.

The importance of high quality RNA for gene expression analysis was described previously [1,2]. However, there are reports that found only small alterations in gene expression in degraded samples, and not in all genes [3]. In the present study, gene expression was analyzed in 13 samples, fresh or frozen, and sorted with different FACS machines. There was no statistical difference between RIN values and threshold cycle. Nevertheless, more stable expression of the reference genes in fresh samples, sorted with a BD Influx, was seen (figure 6.4). Van Rijn *et al* [14] sorted canine healthy pituitary samples previously, and obtained RNA with a quality of >8 RIN. Differences in FACS machine, hydrodynamic forces during sorting, collection method, RNA isolation kit and cell viability prior to FACS, may have resulted in poorer RNA quality in the present study. Due to the value of clinical samples, it is important to know if samples with low RNA integrity are still useful for quantification. Preferably, microarray analysis has to be done on healthy pituitary and pituitary adenoma samples with a different degradation state, to test which genes are alternated by degradation [3]. With this knowledge, samples with low RIN values can be used for qPCR if Ct values are corrected for RNA integrity as described by Romero *et al* [4].

6.5 Cited references

[1] Birdsill AC, Walker DG, Lue L, Sue LI, Beach TG. Postmortem interval effect on RNA and gene expression in human brain tissue. Cell Tissue Bank 2011;12:311-8.

[2] Koppelkamm A, Vennemann B, Lutz-Bonengel S, Fracasso T, Vennemann M. RNA integrity in post-mortem samples: influencing parameters and implications on RT-qPCR assays. Int J Legal Med 2011;125:573-80.

[3] Opitz L, Salinas-Riester G, Grade M, Jung K, Jo P, Emons G et al. Impact of RNA degradation on gene expression profiling. BMC Med Genomics 2010;3.

[4] Gallego Romero I, Pai AA, Tung J, Gilad Y. RNA-seq: impact of RNA degradation on transcript quantification. BMC Biol 2014;30:42.

[5] Houseley J, Tollervey D. The many pathways of RNA degradation. Cell 2009;136:763-76.

[6] Mollet M, Godoy-Silva R, Berdugo C, Chalmers JJ. Acute hydrodynamic forces and apoptosis: a complex question. Biotechnol Bioeng 2007;98:772-88.

[7] Mollet M, Godoy-Silva R, Berdugo C, Chalmers JJ. Computer simulations of the energy dissipation rate in a fluorescence-activated cell sorter: implications to cells. Biotechnol Bioeng 2008;100:260-72.

[8] Riedmaier I, Bergmaier M, Pfaffl MW. Comparison of two available platforms for determination of RNA| quality
 Biotechnol 2010;24:2154-9.

[9] Schroeder A, Mueller O, Stocker S, Salowsky R, Leiber M, Gassmann M et al. The RIN: an RNA integrity number for assigning integrity values to RNA measurements. BMC Mol Biol 2006;7.

[10] Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med 1996;183:1797-806.

[11] van Rijn SJ, Riemers FM, van den Heuvel D, Wolfswinkel J, Hofland L, Meij BP et al. Expression stability of reference genes for quantitative RT-PCR of healthy and diseased pituitary tissue samples varies between humans, mice, and dogs. Molecular neurobiology 2014;49:893-9.

[12] Nishimoto KP, Newkirk D, Hou S, Fruehauf J, Nelson EL. Fluorescence activated cell sorting (FACS) using RNAlater to minimize RNA degradation and perturbation of mRNA expression from cells involved in initial host microbe interactions. J Microbiol Methods 2007;70:205-8.

[13] Eikmans M, Rekers NV, Anholts JD, Heidt S, Claas FH. Blood cell mRNAs and microRNAs: optimized protocols for extraction and preservation. Blood 2013;121:81-9.

[14] van Rijn SJ, Gremeaux L, Riemers FM, Brinkhof B, Vankelecom H, Penning LC et al. Identification and characterisation of side population cells in the canine pituitary gland. Veterinary Journal 2012;192:476-82.

6.6 Supplementary

Protocol	Sample nr.	Tissue	Media	SP/MP	Instrument	Number of sorted cells	Nanodrop quantity (ng/µl)	Nanodrop quality 260/280	Bioanalyzer quantity (pg/µl)	Bioanalyzer quality RIN	Quanitity calculated for 30.000 cells
B) 350 µl RLT-buffer + β-ME; kept on ice											
	39	Pituitary	2A/2B	MP	BD Influx	1567	6,1	1,58	32	1	613
	40	Pituitary	2A/2B	SP	BD Influx	1953	6,5	1,08	17	1	261
	40	Pituitary	2A/2B	MP	BD Influx	12858	8,7	1,65	152	6,7	355
	41	Adenoma*	2A/2B	SP	BD Influx	768	3,9	1,72	5	N/A	195
	41	Adenoma*	2A/2B	MP	BD Influx	12888	20,4	1,5	409	2,2	952
	42	Pituitary	2A/2B	SP	BD Influx	17123	2,6	2,84	63	N/A	110
	42	Pituitary	2A/2B	MP	BD Influx	16329	4,7	4,63	71	4,6	130
	43	Pituitary	2A/2B	SP	BD Influx	1449	18,48	1,39			
	43	Pituitary	2A/2B	MP	BD Influx	14439	22,85	1,39			
	44	Adenoma*	2A/2B	SP	BD Influx	219	3,28	1,20			
	44	Adenoma*	2A/2B	MP	BD Influx	4380	22,92	1,47			
	45	Adenoma*	2A/2B	SP	BD Influx	154	2,57	2,08			
	45	Adenoma*	2A/2B	MP	BD Influx	216	2,42	2,27			
	46	Pituitary	2A/2B	SP	BD Influx	43983	39,4	1,48			
	46	Pituitary	2A/2B	MP	BD Influx	46065	9,76	1,81			
C) 350 µl RLT-buffer + β-ME; snap frozen											
	32	Pituitary	1A/1B	SP	FACSAria II	1800	4,5	1,53	1314	N/A	21900
	32	Pituitary	1A/1B	MP	FACSAria II	7000	5,5	1,34	134	1	574
	33	Pituitary	1A/1B	SP	FACSAria II	10.000	10,1	1,36	65	1	195
	33	Pituitary	1A/1B	MP	FACSAria II	8700	4,1	1,52	553	1,1	1907
	34	Pituitary	1A/1B	SP	FACSAria II	7100	4,8	1,68			
	34	Pituitary	1A/1B	MP	FACSAria II	4000	5,5	2,06			
	35	Pituitary	1A/1B	SP	FACSAria II	2900	7,7	1,69	0	N/A	
	35	Pituitary	1A/1B	MP	FACSAria II	2700	5,9	1,21	0	N/A	
	36	Pituitary	1A/1B	SP	FACSAria II	64	6	3,67	0	N/A	
	36	Pituitary	1A/1B	MP	FACSAria II	2938	5,5	1,45	0	N/A	
D) 350 µl RLT-buffer, back in our own laboratory β-ME is added; kept on ice											
	37	Pituitary	2A/2B	SP	FACSAria II	5981			46	1	231
	37	Pituitary	2A/2B	MP	FACSAria II	30.000			102	1,3	102
E) RNA later; kept on ice; storted in RLT buffer at -70C; 5 min; 3000 rpm											
	6	Adenoma	1A/1B	SP	FACSAria II	6.300	9,5	1,38			
	6	Adenoma	1A/1B	MP	FACSAria II	9.000	4,4	1,86			
	7	Pituitary	1A/1B	SP	FACSAria II	1.915	6,8	2,07			
	7	Pituitary	1A/1B	MP	FACSAria II	1.915	6,8	2,07			
	8	Pituitary	1A/1B	SP	FACSAria II	7.219	7,4	1,48	88	2,9	366
	8	Pituitary	1A/1B	MP	FACSAria II	4.472	12,6	1,6	16	1	107
	9	Pituitary	1A/1B	SP	FACSAria II	4.472	12,6	1,6	16	1	107
	9	Pituitary	1A/1B	MP	FACSAria II	503	4,4	1,2	34	1	2028
	10	Pituitary	1A/1B	SP	FACSAria II	1.800	5,9	1,46			
	10	Pituitary	1A/1B	MP	FACSAria II	618	3	2			
	11	Pituitary	1A/1B	SP	FACSAria II	720	4,2	1,38	72	5,7	3000
	11	Pituitary	1A/1B	MP	FACSAria II	1.200	5,2	1,75	18	1	450
	12	Adenoma	1A/1B	SP	FACSAria II	13.000	8,2	1,67	27	1	62
	12	Adenoma	1A/1B	MP	FACSAria II	66.000	9,6	1,48	75	1,2	34
	13	Adenoma	1A/1B	SP	FACSAria II	28.000	4,7	2,07	100	6,2	107
	13	Adenoma	1A/1B	MP	FACSAria II	14.000	3,9	1,39	0	N/A	
	14	Adenoma	1A/1B	SP	FACSAria II	9.800	4,1	1,8	952	N/A	2914
	14	Adenoma	1A/1B	MP	FACSAria II	5.000	4	1,36	75	5,9	450
	15	Adenoma	1A/1B	SP	FACSAria II	80.000	4,1	1,09			
	15	Adenoma	1A/1B	MP	FACSAria II	51.000	5,5	1,69			
	21	Adenoma	1A/1B	SP	FACSAria II	50.000	3,5	2,13	2708	1	1625
	21	Adenoma	1A/1B	MP	FACSAria II	100.000	6,8	1,28	120	1,1	36
	23	Adenoma	1A/1B	SP	FACSAria II	140.000	8,8	1,77	2158	8,1	462
	23	Adenoma	1A/1B	MP	FACSAria II	150.000	8,5	1,51	7230	N/A	1446

Tabel 6.5: RNA quantity and quality measured on the Nanodrop ND-1000 and Agilent Bioanalyzer 2100 (Agilent technologies) of samples collected by different protocols.

Protocol	Sample nr.	Tissue	Media	SP/MP	Instrument	Number of sorted cells	Nanodrop quantity (ng/µl)	Nanodrop quality 260/280	Bioanalyzer quantity (pg/µl)	Bioanalyzer quality RIN	Quanitity calculated for 30.000 cells
G1) Empty, ice cold 1 ml tube; kept on ice; 5 min; 3000 rpm											
	37	Pituitary	2A/2B	SP	FACSAria II	3677			49	1	400
	37	Pituitary	2A/2B	MP	FACSAria II	25.000			63	6,5	76
G2) Empty, ice cold 1 ml tube; kept on ice; 10 min; 3000 rpm											
	38	Pituitary	2A/2B	SP	FACSAria II	245	5,2	1,21			
	38	Pituitary	2A/2B	MP	FACSAria II	5600	5,7	1,31			
G3) Empty, ice cold 1 ml tube; kept on ice; 10 min; 2000 rpm											
	38	Pituitary	2A/2B	MP	FACSAria II	4300	7,1	1,54			

Table 6.5 (continuation): RNA quantity and quality measured on the Nanodrop ND-1000 (Isofen Life Sciences) and Agilent Bioanalyzer 2100 (Agilent technologies) of samples collected by different protocols. Samples are numbered the same as the samples depicted in chapter 5. All healthy pituitary adenoma samples are canine, except for the marked (*) feline adenomas. Medium 1A consisted of 1 liter DMEM 1:1/F12 containing 5 mg transferrin, 5 mg Insulin, 1% penicillin streptomycin, 1 mg catalase and 1 g NaHCO3. Medium 1B was HBSS, containing 2% foetal calve serum(FCS), 2µg/ml propidium iodide (PI) , 0.4% DNase and 0.025% EDTA. Medium 2A consisted of DMEM Glutamax, 2% FCS+ 10 mM Hepes + 0.4% Insulin and medium 2B was HBSS enriched with 2% FCS and 2µg/ml PI. FACS analysis was performed on a FACSaria II (SORP) at the Hubrecht institute in Utrecht, or on a BD Influx at the department of Immunology, Faculty of Veterinary Medicine, Utrecht University. SP, side population; MP, main population; β-ME, β-mercaptoethanol;

7. The role of PC2 and Pax7 in pituitary adenomas

7.1 Introduction

The pituitary gland is a small endocrine gland located in the pituitary fossa, and consists of three functional units, the anterior lobe (pars infundibularis and pars distalis of the adenohypophysis), the intermediate lobe (pars intermedia) and posterior lobe (neurohypophysis)[1]. In humans, the adult pituitary does not have an IL because this tissue regresses after the 15th week of gestation [2]. Corticotroph cells produce ACTH and are located in both the anterior lobe and the intermediate lobe, while melanotroph cells are located in the intermediate lobe and produce α-MSH. Pituitary tumors can arise from both corticotroph or melanotroph cells [3]. Corticotroph and melanotroph cells arise from the POMC cell lineage, that develops under influence of the transcription factor Tpit [4]. In corticotrophs, the prohormone POMC is processed in ACTH by the peptide prohormone convertase 1 (PC1). In melanotrophs, PC1 cleaves POMC in ACTH, followed by cleavage of ACTH into α-MSH by prohormone convertase 2 (PC2)[2]. In a study of Iino *et al* [5], approximately 50% of the ACTH-producing pituitary adenomas in humans stained positive against PC2, whereas healthy pituitaries did not. Plasma α-MSH was not detected in PC2-negative cases or in normal controls, though four out of five patients with detectable plasma α-MSH had PC2 positive adenomas, indicating for an intermediate lobe origin in a subset of corticotroph adenomas. Since it was found that 80% of the macroadenomas expressed PC2, Iino *et* al [5] suggest a possible relationship of PC2 expression and tumor growth. Another gene possibly related to tumor growth, is thrombospondin-2 (Thbs2). Thbs2 is an extracellular matrix protein with anti-angiogenic activity. Treatment of the human pituitary cell line (HP75) with TGF-β up regulated Thbs2 expression and reduced pituitary cell growth [6].

Regulation of ACTH secretion includes the release of corticotropin releasing factor (CRF) and the mesolimbic dopamine system [7]. The Dopamine D2 receptor (Drd2) is expressed in the lactotropes and melanotropes of the pituitary gland and inhibits PRL and α-MSH-secretion, respectively [8]. Approximately 75% of the corticotroph adenomas express Drd2 and the dopamine agonist cabergoline significantly inhibits ACTH secretion in vitro and in vivo [9]. Occhi *et al* showed that up regulation of Drd2 in the pituitary adenoma cell line (AtT20) enhanced paired box 7 (Pax7) expression significantly, but not PC2 expression [10].

Pax7 is a member of the Pax transcription factor family and a so-called selector gene. A selector gene is defined as a cell-autonomously acting gene that provides early patterning and positional identity [2]. Although differentiation of the POMC lineage into corticotroph or melanotroph cells requires Tpit, Pax7 is critical for activation of melanotroph specific genes and repression of corticotroph specific genes [2]. Budry *et al* [2] showed that Pax7 inactivation results in a decrease of mRNA expression of POMC, PC2 and Drd2, and Pax7$^{-/-}$ knockout mice showed a corticotroph gene expression profile. Restricted expression of Pax7 to the IL was confirmed by immunohistochemistry [2]. Besides, Hosoyama *et al* [11] showed in mice that around 60% of ACTH staining cells in the intermediate lobe also expressed Pax7, indicating for a melanotroph feature in pituitary adenomas. In canine pituitary adenomas, 34% of the adenomas showed Pax7 expression and, contrary to the study of Budry *et al*, one out of five healthy pituitaries stained positive. Besides, not all intermediate lobe adenomas showed Pax7 expression (van Rijn *et al* 2014, unpublished).

Like Pax7, SRY (sex determining region Y)-box 2 (Sox2)is a transcription factor which plays a role in developmental processes in the pituitary gland. In the postnatal pituitary, $Sox2^+$ cells are located in Rathke's pouch and in clusters scattered throughout the parenchyma [12]. $Sox2^+$ cells show several stem cell characteristics [12] and in a study of Kelberman *et al* [13], abnormal morphogenesis of the pituitary was found in one-third of the $Sox2^{+/-}$ heterozygous mice, whereas homozygous deletion of Sox2 was lethal. Besides, $Sox2^+$ cells do give rise to pituitary adenomas in transgenic mice [14], indicating for a possible role of Sox2 in pituitary tumorigenesis. However, in canine pituitary adenomas, no significant difference was found in Sox2 protein expression between healthy pituitaries and pituitary adenomas, and Sox2 expression was not correlated with clinical signs (van Rijn *et al*, unpublished).

Since it is known that Pax7 expression is limited to the intermediate lobe in mice, Pax7 expression in canine pituitary adenomas has to be further investigated to explain the results found by van Rijn *et al* (unpublished). The goal of the present study was (1) to search for an association between Pax7 and PC2 protein expression in canine pituitary adenomas, and (2) to determine mRNA expression of Pax7, its related genes Drd2, POMC and PC2, as well as Sox2 and Thbs2 by means of quantitative PCR analysis.

7.2 Methods

7.2.1 Experimental design

Immunohistochemistry for PC2 was done on 2 canine healthy pituitaries , and 3 pituitary adenomas. To investigate the gene expression of Pax7 and its related genes, qPCR for Pax7, Sox2, PC2, Drd2, Thbs2 and POMC was performed on 20 canine healthy pituitary and 20 canine pituitary adenoma samples. PC2 protein expression was compared with the Pax7/Sox2 results of the study of van Rijn *et al* (unpublished). Besides, the gene expression profile was correlated with clinical data like recurrence, P/B ratio and plasma ACTH and α-MSH.

7.2.2 Tissue sampling

PDH was diagnosed based on clinical biochemistry of blood, hormonal and urine parameters as described previously [15,16]. Computed tomography (CT) was used to localize the tumor and to determine the P/B-ratio [17]. Tumors with a P/B-ratio of >0,31 were defined as enlarged pituitaries. Pituitary adenomas were removed by transsphenoidal hypophysectomy and collected for research purposes after the owner's consent. The diagnosis PDH was confirmed by immunostaining for ACTH, α-MSH and GH. Healthy pituitary samples were obtained from healthy dogs euthanized in unrelated experiments and approved by the Ethics Committee on Animal Experimentation of the Faculty of Veterinary Medicine, Utrecht University, the Netherlands. Directly after euthanasia the skull was opened, the brain removed and the pituitary collected.

Samples for immunohistochemistry: Specimens of 3 pituitary adenomas and 2 healthy pituitaries were fixed in 4% methanol-formaldehyde, embedded in paraffin and cut in sections of 4 µm thick. All pituitary adenomas were obtained from patients diagnosed with PDH. Healthy pituitaries were obtained from 2 healthy Beagles. As a positive control, slides of canine healthy testis was used, kindly provided by Monique van Wolferen (Faculty of Veterinary Medicine, Utrecht University).

Samples for qPCR: All pituitary adenomas were obtained during transsphenoidal hypophysectomy from patients with confirmed Cushing's disease. Samples were snap frozen in liquid nitrogen and stored at -70°C before further processing. In total, 20 pituitary adenomas and 20 healthy pituitaries were obtained and used in the study. Clinical characteristics, histopathological diagnosis and follow-up information of the patients included in the qPCR study are listed in table 7.1.

7.2.3 Immunohistochemistry

Sections were deparaffinized and rehydrated through a series of xylene (Klinipath B.V., Duiven, the Netherlands) 96% ethanol (Boom B.V., Meppel, the Netherlands) 80% ethanol, 70% ethanol and 60% ethanol. For optimization of the protocol, different antigen retrieval methods were used. Antigen retrieval was performed by incubation in citrate (10mM Citric acid pH=6,0) (Merck KGaA, Germany) or Tris EDTA (10mM Tris Base (Merck, Germany), 1mM EDTA Solution (Sigma-Aldrich), pH 9.0) placed in a water bath (98°C) during 15 and 20 minutes, respectively. Slides were left to cool down for 60 minutes at RT. Besides, antigen retrieval was performed with the enzymes pronase (0.05% in PBS)(Merck, Germany) and proteinase K (Dako s3004). Slides were incubated at 37°C for 15 minutes with pronase, and for 3 and 6 minutes with Proteinase K. Following antigen retrieval, slides were washed twice. All wash steps were performed with with Tris-buffered saline +0.1%Tween (TBST) (Tween20, Boom B.V., Meppel, the Netherlands) on a mechanical shaker. Slides were incubated for 10 minutes at RT with endogenous enzyme block (Dako s2003), washed twice, followed by a 60 minute incubation with 10% normal goat serum (NGS). Incubation with the primary antibody took place overnight at 4°C. The anti-PC2 polyclonal rabbit antibody (Bioss, Isotype IgG) was diluted in 5% NGS. The following day, slides were washed 3 times and incubated for 30 minutes at RT with the secondary antibody , labeled polymer-HRP anti-rabbit (Dako Envision Systems). After 3 wash steps, slides were stained with 3,3'-diaminobenzidine (DAB) substrate (Dako) for 15 minutes, washed and counterstained with hematoxylin (Vector Laboratories, Inc., Burlingame, CA) for 10 seconds. Slides were washed for 10 minutes with running tap water, hydrated in a series of 60% alcohol, 70% alcohol, 80% alcohol, 2 times 96% alcohol, 100% alcohol, 2 times xyleen, and covered with permanent mounting medium (Vector Laboratories).

7.2.4 qPCR

Total RNA was isolated from snap frozen samples, using Qiagen RNeasy Mini Kit (Qiagen, Leusden, the Netherlands) according to the manufacturer's instructions. RNA quantity and quality was measured with the Agilent BioAnalyzer 2100 and the RNA integrity number (RIN) values were above 7.0, indicating for sufficient RNA quality to perform qPCR. cDNA was synthesized using the iScript™ cDNA Synthesis Kit (Bio-rad, Veenendaal, the Netherlands) according to manufacturer's instructions. The RT-qPCR reaction was performed in duplicate on a BioRad I-Cycler (Bio-Rad). For each qPCR sample, a total volume of 10 µL was used, containing 5.0 µL IQ SYBR green SuperMix (Bio-Rad), 4.0 µL of 50-fold diluted cDNA, and 100 µM of both forward and reverse primers. For primers that were not previously described, primer sets were developed and tested with a temperature gradient to determine the optimal annealing temperature (T_a). Details of the primers used are depicted in Table 7.2. To normalize gene expression the reference genes *tbp, hmbs* and *ywhaz* were used, previously identified as the most stable reference genes for pituitary and adenoma samples [18].

no.	age (y)	Seks	Weight (kg)	Breed	UCCR x10^-6[a]	Dexa-methasone supression[b]	Pituitary size (H x W x L) [c]	P/B ratio[d]	Histopathological diagnosis[e]	Immuno-histochemistry[f]	Survival (mo)	DFI	Remission[g]	Recurrence[h]	ACTH (pmol/L)	A-MSH (pmol/L)
21	8,8	M*	24,7	Irish Terrier	29,5	93,6	5x7x6	0,3	Adenoma	N/A	19,8	N/A	No	No	122,5	50
22	8,6	M	33,4	Boxer	44,9	92,4	12,4x11,6x11	0,7	Adenoma	N/A	28,1	23,8	No	Yes	60,5	11
23	8,1	V*	23	Crossbred	20	80	7x7,7x11,3	0,35	Adenoma	N/A	67,3	28,1	No	Yes	92	15,5
24	9,8	M*	8,1	Dashund	19,75	92,9	7,4x6x6,6	0,54	Adenoma	N/A	0,5	N/A	N/A	N/A	168,5	8,5
25	11,7	M*	13	Jack Russell Terrier	13,5	74,1	12,6x13,2x16,2	0,74	Adenoma	ACTH en GH +	20,6	8,4	Yes	Yes	81,5	12
26	10,4	V*	3,7	Maltezer	213,5	67,2	13,8x14,4x15,3	0,91	Adenoma	ACTH +	0,1	N/A	N/A	N/A	663	366
27	10,9	V*	8,5	Jack Russell Terrier	26	88,1	6,3x7,1x8,7	0,38	Adenoma	ACTH + GH +-	18,6	18,6	No	Yes	294,5	7
28	12,1	M	24	Beagle	N/A	N/A	22,5x21,3x20,7	1,25	Adenoma	ACTH en MSH +	0,0	N/A	N/A	N/A	141	20
29	8,7	V*	35	Boxer	49,2	98,8	10,1x10,7x10,8	0,53	Adenoma	ACTH en MSH +	7,9	7,9	N/A	no	N/A	N/A
30	10	V*	28,5	Golden Retriever	31	-12,9	10x11,4x10,5	0,56	Adenoma	ACTH en MSH +, GH -	40,8	40,8	Yes	No	149	16
31	14	M*	21	Vizsla	N/A	N/A	15x21,4x18,3	0,98	Adenoma	ACTH en MSH +, GH -	13,2	13,2	Yes	No	43	10
32	10,8	V*	31	German Shorthaired Pointer	22,1	99,1	5,8x8x7	0,32	Adenoma	ACTH en MSH +, GH -	14,8	14,8	Yes	No	46	7
33	6,7	V	48	Berner Senner	100	61	11,7x13x11,7	0,69	Adenoma, mal. Char.	ACTH + aMSH +- GH -	23,8	23,8	Yes	No	191	4
34	9,4	M	34,5	Crossbred labrador	19,7	81,7	7,1x9,1x9,5	0,44	Adenoma	ACTH en MSH +	73,4	73,4	Yes	No	53	13
35	10,4	M*	5,4	Crossbred	62,5	69,6	5,3x5,8,6	0,38	Adenoma	ACTH en MSH +	4,3	4,3	Yes	No	N/A	N/A
36	8,3	V*	36,5	Labrador Retriever	30,7	88,3	11,5x11,8x11,6	0,56	Pars intermedia	N/A	84,4	84,4	No	Yes	130	35
37	5,9	V	24,7	Nova scotia duck tollington retriever	82,4	93,7	8x9x8,4	0,49	Adenoma	ACTH +	67,1	29,8	Yes	Yes	115	7
38	8,2	M	23,2	Beagle	74,5	75,8	16,6x14,6x14	1	Adenoma	ACTH en MSH -	61,7	40,1	Yes	Yes	139	22
39	3,7	M*	6,7	Miniature pincher	18,6	N/A	5x6,2x8	0,37	No adenoma	ACTH en MSH -	50,7	38,5	Yes	Yes	54	4
40	6,5	M	12,8	Crossbred	81	60,5	13,8x16,1	0,99	Adenoma	N/A	18,8	4,4	Yes	No	84	5

Table 7.1: Clinical characteristics, histopathological diagnosis and follow-up information of the patients included in the quantitative PCR study. Abbreviations: *, castrated; F, female; M, male, N/A, not available, UCCR, urinary corticoid-creatinine ratio; *, alive; +, marked immunoreactivity, +/-, weak immunoreactivity, -, no immunoreactivity; DFI, disease-free interval. [a] Preoperatieve urinary-creatinine ratio (reference < 10×10^{-6}); values are the mean of 2 morning urine samples with a 1-d interval., [b] Preoperative degree of UCCR suppression after high-dose dexamethasone, [c] Pituitary size in mm, as measured on preoperative helical computed tomography, [d] P/B$\times10^2$ (mm^{-1}), ratio of the pituitary size and the brain area. P/B ≤ 0.31 indicates a non-enlarged pituitary; P/B > 0.31 indicates an enlarged pituitary. ; [e,f] Diagnosis as stated by a veterinary pathologist based on hematoxylin and eosin staining and immunohistochemistry for ACTH, α-MSH, and GH. [g] Remission was defined as UCCR < 10 x 10^{-6} and resolution of clinical signs of hypercortisolism. [h] Recurrence was defined as UCCR ≥ 10 x 10^{-6} and return of clinical signs and symptoms of hypercortisolism after initial remission.

Gene	NCBI Ref. seq.	F/R	Sequence	Exon	Amplicon size	T_a (°C)
Drd2	XM_005619479	F	5' CCACTCAAGGGCAACTG-'3	5/6	87	61
		R	5'-CCTGTTCACTGGGAAACTC-'3	6		
hmbs	XM_546491	F	5'-TCACCATCGGAGCATCT-'3	6	112	61
		R	5'-GTTCCCACCACGCTCTTCT-'3	7		
Pax7	XM_005617934	F	5'-AAGGACGGACACTGTGAC-'3	3	82	58
		R	5'-CTTTCTTCCCGAACTTGATTCTG-'3	4		
PC2	XM_542880	F	5'-ATTCAACAGAAAGAAGCGGG-'3	3	141	62
		R	5'-TCTGCCACATTCAAATCCAG-'3	4		
POMC	XM_844370	F	5'-GCCTGCAAGCCCGACCTCTC-'3	3	178	62
		R	5'-CTCCGCCCGCCGCCACCTCTTCTT-'3	3		
Sox2	XM_005639752	F	5'-AACCCCAAGATGCACAACTC -'3	1	152	61
		R	5'-CGGGGCCGGTATTTATAATC-'3	1		
tbp	XM_849432	F	5'-CTATTTCTTGGTGTGCATGAGG-'3	5	96	58
		R	5'-CCTCGGCATTCAGTCTTTTC-'3	5		
Thbs2	XM_541204	F	5'-CTCGGGACAGTCACTTCAG -'3	3	84	62
		R	5'-CTTTCTTGCTCAGAACATCTTCC-'3	3		
ywhaz	XM_533072	F	5'-CGAAGTTGCTGCTGGTGA -'3	2	94	58
		R	5'-TTGCATTTCCTTTTTGCTGA-'3	3		

Table 7.2: Nucleotide sequences, exon, amplicon size and annealing temperature (T_a) of the primers used for RT-qPCR of the genes Dopamine receptor D2 (Drd2), hydroxymethylbilane synthase (hmbs), Paired box 7 (Pax7), Prohormone converstase 2 (PC2), Proopiomelanocortin (POMC), SRY (sex determining region Y)-box 2 (Sox2), tata box binding protein (tbp), Thrombospondin 2 (Thbs2) and ,tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (ywhaz). Abbreviations: F, forward primer; R, reverse primer;

7.2.5 Statistical analysis

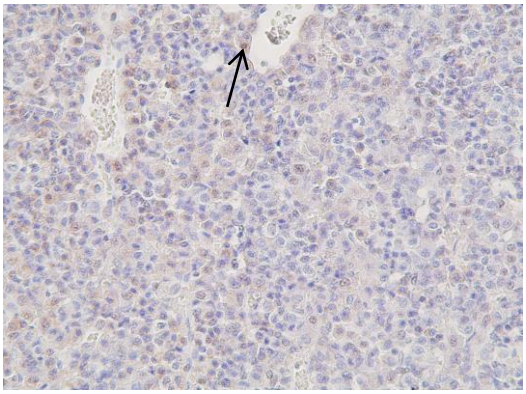
Gene expression profiles were compared between the adenoma and pituitary gland group and between recurrence and non-recurrence group. Recurrence was considered in dogs that went in complete remission, but with return of clinical signs of PDH and a UCCR of $10x10^{-6}$ or higher [15]. Normality was tested with a Shapiro-Wilk test. The Mann-Whitney U-test (PC2, Drd2, Thbs2 and POMC) and T-test (Pax7, Sox2) were performed to determine significant differences between the groups. Correlation among the genes, between gene expression and plasma ACTH and α-MSH, and between gene expression and P/B ratio was investigated using a Spearman's Rho test. Significance was set at $P < 0.05$.

7.3 Results

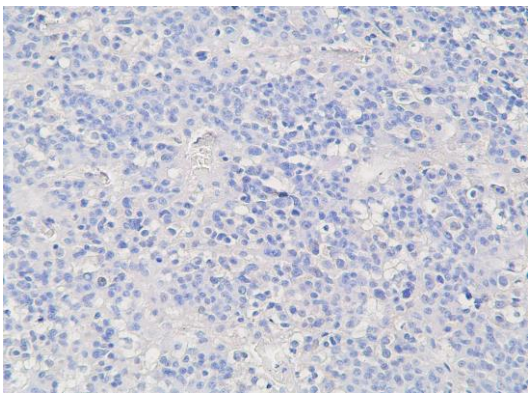
7.3.2 Immunohistochemistry

Different antibody dilutions and antigen retrieval methods were used to optimize the PC2-staining. Without antigen retrieval, aspecific and nuclear staining was seen in healthy pituitary slides using antibody dilutions of 1:100 – 1:400, and no staining was seen when stained with a 1:800 diluted antibody. Cytoplasm staining was seen in Leydig cells of canine healthy testis (1:400 and 1:250 dilution), but no aspecific staining. Enzymatic antigen retrieval with pronase and proteinase K resulted in extensive aspecific staining in the healthy pituitary. Antigen retrieval by incubation with Tris-EDTA resulted in less aspecific staining, but nucleus staining was seen. After incubation with citrate, aspecific and nucleus staining was seen in a 1:250 dilution (figure 7.1A), but not in a 1:400. antibody dilution. In total, slides of 2 different healthy pituitaries, and of 3 pituitary adenomas were

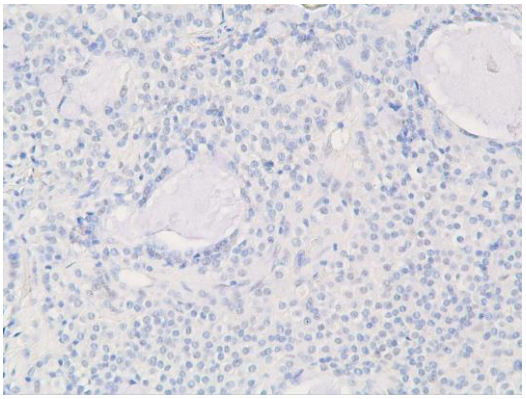
stained in a 1:250 antibody dilution, after incubation with citrate (15 min, 98°C). Two adenomas used in the present study did not stain positive for Pax7 in the study previously performed by van Rijn *et al* (unpublished) whereas one adenoma did. In all adenoma slides, no PC2 positive cells were found (figure 7.1B-C). In the positive control, Leydig cells of the testis stained positive using the same protocol (figure 7.1D).



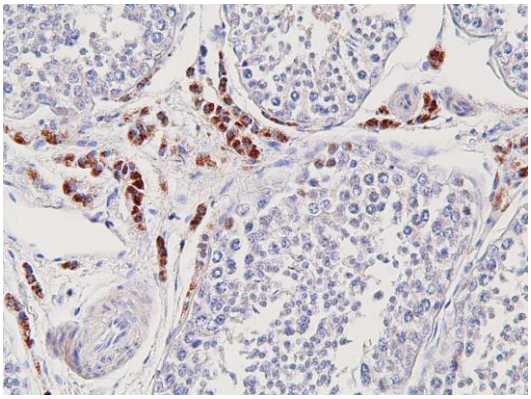
(A) Canine healthy pituitary



(B) Canine pituitary adenoma (Pax7-)



(C) Canine pituitary adenoma (Pax7+)



(D) Canine healthy testis

Figure 7.1: (A) In the canine healthy pituitary, aspecific staining, including nucleic staining (arrow) was seen. **(B,C)** No PC2 immunoreactivity was seen in canine pituitary adenomas. **(D)** Leydig cells of the canine testis stain positive (arrow). All slides were incubated for 15 minutes in citrate (98°C) and stained with anti-PC2 polyclonal rabbit antibody (Bioss, Isotype IgG) in a 1:250 dilution. Magnification of 200x for each figure.

7.3.3 qPCR

To address levels of mRNA levels of the genes Pax7, Sox2, PC2, Drd2, Thbs2 and POMC, qPCR was performed in 20 pituitary and 20 adenoma samples. Delta Ct (dCt) values were calculated with the mean of the reference genes *tbp*, *hmbs* and *ywhaz* (figure 7.2). Relative mRNA expression was down regulated in the adenoma samples for all genes and is significantly for Pax7, Sox2, PC2, Drd2 and Thbs2 (P = 0.001 for Pax7; P = 0.005 for Sox2; P = 0.003 for PC2; P <0.001 for Drd2; and P = 0.030 for Thbs2). There was no significant difference between the group with recurrence (n=5) and the group without recurrence (n=11). A significant correlation was found between Pax7 and PC2 (P < 0.001, correlation coefficient (CC) = 0.744)(Figure 7.3A) and between Pax7 and POMC (P = 0.030, CC =

0.511) in the healthy pituitary samples. In the adenoma samples, a significant correlation was found between Pax7 and Sox2 (P = 0.014, CC = 0.566), Pax7 and Drd2 (P = 0.008, CC = 0.605)(figure 7.3B), and between Drd2 and PC2 (P < 0.001, CC = 0.736).

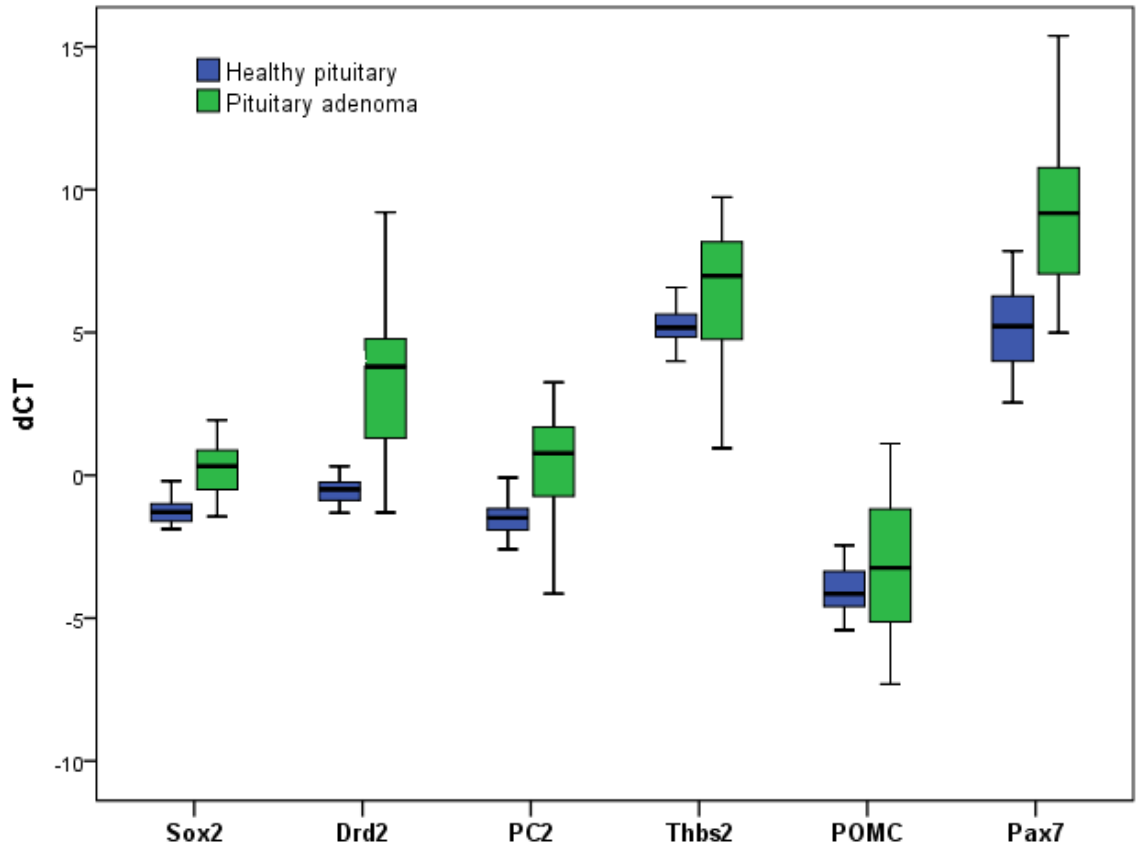


Figure 7.2: Boxplots of the dCT values of both groups (healthy pituitary, blue; pituitary adenoma, purple) for SRY (sex determining region Y)-box 2 (Sox2), Dopamine receptor D2 (Drd2), Prohormone converstase 2 (PC2),)Thrombospondin 2 (Thbs2), Proopiomelanocortin (POMC), Paired box 7 (Pax7) in healthy pituitaries and pituitary adenomas. dCT values were calculated with the use of the mean of the reference genes tata box binding protein (tbp), hydroxymethylbilane synthase (hmbs) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (ywhaz). In healthy pituitaries, the mean (±SD) dCT value for Pax7 was 5.27±1.94, for Sox2 -1.27±0.43, for PC2 -1.31±1.08, for Drd2 -0.35±0.98, for Thbs2 5.03±1.13 and for POMC -4.12±1.24. In pituitary adenomas, the mean (±SD) dCT value for Pax 7 was 8.58±3.44, for Sox2 0.19±0.91, for PC2 0.71±2.57, for Drd2 3.28±2.71, for Thbs2 6.44±2.48 and for POMC -3.10±2.47. dCT values significantly differed between healthy pituitary and pituitary adenoma for Pax7, Sox2, PC2, Drd2 and Thbs2 (P=0.001 for Pax7; P=0.005 for Sox2; P=0.003 for PC2; P <0.001 for Drd2; and P=0.030 for Thbs2), but not for POMC (P=0.168).

Of the 20 patients included in the qPCR study, P/B ratio, plasma ACTH (pmol/L) and α-MSH (pmol/L) were measured. Mean (±SD) P/B ratio was 0.62±0.06, mean (±SD) ACTH was 145.97±33.80 and mean (±SD) α-MSH was 34.06±19.72. A significant correlation was found between the P/B ratio and Sox2 (P=0.028, CC=0.491), Drd2 (P=0.043, CC=0.456), Thbs2 (P = 0.032, CC = -0.48) and POMC (P = 0.037, CC = 0.468). No significant difference was found between recurrence and gene expression. Comparing the expression profiles of the genes with plasma ACTH and α-MSH, no significant correlation was found.

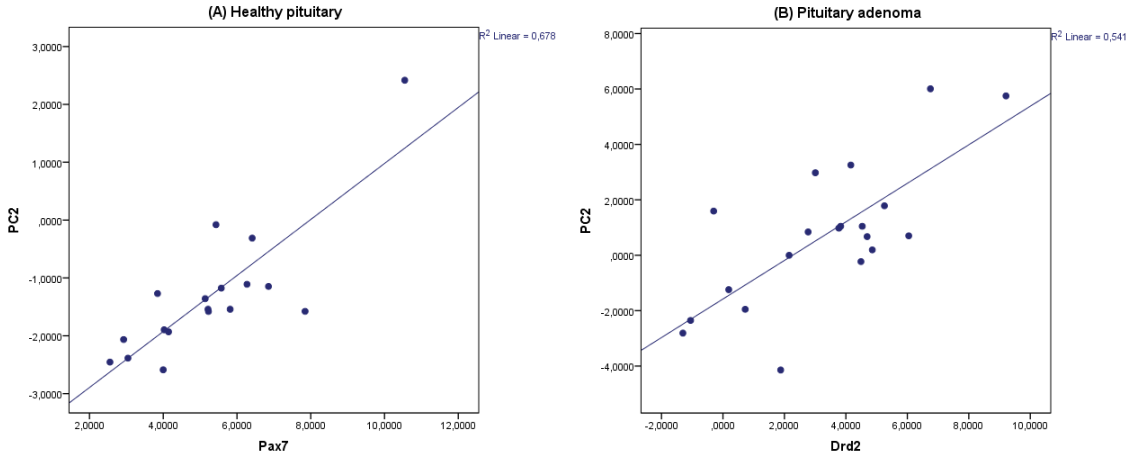


Figure 7.3: (A) Correlation between Paired box 7 (Pax7)(x-axis) and Prohormone converstase (PC2)(y-axis) in the healthy pituitary ($P < 0.001$). The correlation coefficient was 0.744. **(B)** Correlation between Dopamine receptor d2 (Drd2)(x-axis) and PC2 (y-axis) in pituitary adenomas ($P < 0.001$). The correlation coefficient was 0.736.

7.4 Discussion

In the present study, PC2 protein expression in the canine pituitary gland and pituitary adenoma was investigated and compared with Pax7 expression, as investigated previously (van Rijn *et al*, unpublished). It was hypothesized that Pax7 immunopositivity was linked to an intermediate lobe origin of the pituitary adenoma. Since intermediate lobe adenomas tend to be larger, they are thought to have a worse prognosis than anterior lobe adenomas [15,19,20]. In previous studies, Pax7 expression was found in human and canine corticotroph pituitary adenomas [2,11], suggesting a possible role for Pax7 in the development of these adenomas. It had been reported that Pax7 expression is restricted to the melanotrophs. However, van Rijn *et al* (unpublished) found one healthy pituitary to be positive for Pax7 (15.2%), and not all intermediate lobe adenomas stained positive. Since it is known that in humans the intermediate lobe regresses after the 15th week of gestation [2], this is a remarkable finding. However, no research is done about the embryogenesis of the intermediate lobe of the canine pituitary.

To test whether Pax7 immunoreactivity in the healthy pituitary was due to intermediate lobe infiltration, and to further explore the melanotroph origin of the investigated adenomas, PC2 staining was performed. PC2 cleaves ACTH in α-MSH and is expressed in melanotroph cells of the pituitary gland. Biosynthesis of the inactive precursor of PC2 occurs in the endoplasmic reticulum (ER). The protein 7B2 binds to pro-PC2, facilitates the transport through the Golgi network in secretory granules, and activates pro-PC2 [21]. Since PC2 is processed and activated in granulae in the cytoplasm, the nucleus staining we found in the slides, was due to aspecific staining. Different antibody dilutions and antigen retrieval methods were used, but no PC2 positive cells were seen in the healthy pituitary and pituitary adenoma slides (figure 7.1A,B). Also, one canine pituitary adenoma that did show immunoreactivity for Pax7 (van Rijn *et al*, unpublished) did not have PC2 positive cells (figure 7.1C). However, Leydig cells in the testis of the positive controls did stain positive for PC2 (7.1D). These findings indicate that there is no PC2 protein expression in the canine healthy pituitary and pituitary adenoma. However, Iino *et al* [5] found PC2 expression in 50% of the

human pituitary adenomas, and not in healthy pituitaries. We investigated PC2 expression in only 3 adenomas and a larger subset of samples is needed to exclude PC2 expression in canine pituitary adenomas.

PC2 gene expression was determined by qPCR in both healthy pituitaries and pituitary adenomas, with less PC2 expression in pituitary adenomas (figure 7.2). Iino *et al* [5] suggested for a possible role of PC2 in tumor growth and aggressiveness. In our study, no correlation was found between PC2 gene expression and P/B ratio, but a significant correlation was found between P/B ratio and expression of the anti-angiogenic protein Thbs2, with a down regulation of Thbs2 in larger adenomas. These findings indicate that tumors with increased Thbs2 expression, might be less aggressive. However, no significant difference was found between recurrence and Thbs2 or PC2 expression.

Budry *et al* [2] showed in a study with $Pax7^{-/-}$ knockout mice, that PC2, Drd2 and POMC are Pax7 inducible. The present study shows a correlation of Pax7 with PC2 and POMC in healthy pituitaries, and of Pax7 with Drd2 and Sox2, in pituitary adenomas. Down regulation of Drd2 gene expression was found in pituitary adenomas and, unexpectedly, of PC2. Drd2 and PC2 are both expressed in melanotroph cells [2,22] and PC2 cleaves ACTH into α-MSH [2], whereas Drd2 inhibits α-MSH secretion [8]. The concentration of α-MSH may reflect aggressiveness of the tumor and in most dogs with elevated plasma α-MSH, disease recurred [15]. In the present study, no correlation was found between down regulation of PC2 or Drd2 in adenomas and variations in plasma α-MSH, or recurrence of the disease. However, a correlation was found between PC2 and Drd2 in adenomas, suggesting for a possible co-expression of these proteins to reduce α-MSH secretion in adenomas.

There was no significant difference in expression of POMC, the precursor of ACTH, between healthy pituitaries and pituitary adenomas. An up regulation was expected, as plasma ACTH levels are elevated in patients with PDH. Multiple signals act together to activate POMC gene expression and they include CRH, cytokines, AVP, catecholamines and vasoactive intestinal peptide [23]. Glucocorticoids inhibit POMC gene expression, though it is known that corticotroph adenomas are relative resistant to the negative feedback of cortisol on ACTH secretion. To further investigate POMC expression in adenomas, protein expression has to be determined, as well as expression of PC1. PC1 cleaves POMC in ACTH, and rapid post-translational protein processing might be a reason for elevated plasma ACTH levels, and not elevated POMC gene expression. On the other hand, down regulation of the glucocorticoid (GC) receptor may induce resistance to the negative feedback, leading to excessive ACTH release. It has been shown that 50% of the GC-receptor resistance pituitary adenomas were deficient in gene expression of the GC protein [24]. However, down regulation was not seen in all pituitary adenomas, and even up regulation of the GC-receptor was found in some adenomas [24]. Therefore, expression of the GC-receptor has to be investigated in the canine pituitary adenoma.

Sox2 is known to play an important role in pituitary development [13,25], where $Sox2^{+}$ cells show several stem cell characteristics [12,14,26]. It is thought that stem cells also play a role in tumorigenesis in the pituitary gland [14]. Andoniadou *et al* [14] showed that $Sox2^{+}$ cells do give rise to pituitary adenomas in transgenic mice, however they suggest $Sox2^{+}$ cells play a paracrine role in tumorigenesis. In the canine pituitary tumor, no differential protein expression of Sox2 could be identified between the healthy pituitary and adenoma (van Rijn *et al*, unpublished). However, we

found a significant difference in Sox2 gene expression, with higher expression in the canine healthy pituitary. Besides, a correlation between Pax7 and Sox2 was found in pituitary adenomas.

The aims of the present study were to search for an intermediate lobe origin in canine pituitary adenomas, which may explain the Pax7 results of the study of van Rijn *et al* (unpublished), and to further investigate Pax7 and its associated genes. PC2 cleaves POMC in ACTH and expression of the protein is thought to be in melanotroph cells of the intermediate lobe of the pituitary gland. However, no PC2 protein expression was found in healthy pituitaries and pituitary adenomas. In mice, it has been shown that the genes POMC, Drd2 and PC2 are Pax7 inducible [2], and we found the same results in our study. Correlations were found between Pax7 and the genes in either healthy pituitary samples or pituitary adenomas. Comparing healthy pituitaries with pituitary adenomas, gene expression of Pax7, Drd2, PC2 and Sox2 were remarkably down regulated in pituitary adenomas. The results found in this study cannot support in an explanation for the Pax7 results of the study of van Rijn *et al* (unpublished.), but evoked new research questions to further study tumorigenesis in ACTH-producing canine pituitary tumors.

7.5 Cited references

[1] van Rijn SJ, Tryfonidou MA, Hanson JM, Penning LC, Meij BP. Stem cells in the canine pituitary gland and in pituitary adenomas. Vet Q 2013;33:217-24.

[2] Budry L, Balsalobre A, Gauthier Y, Khetchoumian K, L'honoré A, Vallette S et al. The selector gene Pax7 dictates alternate pituitary cell fates through its pioneer action on chromatin remodeling. Genes & development 2012;26:2299-310.

[3] Rijnberk A KH. Clinical endocrinology of dogs and cats. 2010.

[4] Lamolet B, Pulichino AM, Lamonerie T, Gauthier Y, Brue T, Enjalbert A et al. A pituitary cell-restricted T box factor, Tpit, activates POMC transcription in cooperation with Pitx homeoproteins. Cell 2001;104:849-59.

[5] Lino K, Oki Y, Yamashita M, Matsushita F, Hayashi C, Yogo K et al. Possible relevance between prohormone convertase 2 expression and tumor growth in human adrenocorticotropin-producing pituitary adenoma. J Clin Endocrinol Metab 2010;95:4003-11.

[6] Horiguchi H, Jin L, Ruebel KH, Scheithauer BW, Lloyd RV. Regulation of VEGF-A, VEGFR-I, thrombospondin-1,-2, and-3 expression in a human pituitary cell line (HP75) by TGFβ1, bFGF, and EGF. Endocrine 2004;24:141-6.

[7] Panagiotakopoulos L, Neigh GN. Development of the HPA axis: where and when do sex differences manifest? Frontiers in neuroendocrinology 2014.

[8] Garcia-Tornadú I, Perez-Millan MI, Recouvreux V, Ramirez MC, Luque G, Risso GS et al. New insights into the endocrine and metabolic roles of dopamine D2 receptors gained from the Drd2$^{-/-}$ mouse. Neuroendocrinology 2010;92:207-14.

[9] Pivonello R, Ferone D, de Herder WW, Kros JM, Del Basso de Caro, M.L., Arvigo M et al. Dopamine receptor expression and function in corticotroph pituitary tumors. J Clin Endocrinol 2004;89:2452-62.

[10] Occhi G, Regazzo D, Albiger NM, Ceccato F, Ferasin S, Scanarini M et al. Activation of the Dopamine Receptor Type-2 (DRD2) Promoter by 9-Cis Retinoic Acid in a Cellular Model of Cushing's Disease Mediates the Inhibition of Cell Proliferation and ACTH Secretion Without a Complete Corticotroph-to-Melanotroph Transdifferentiation. Endocrinology 2014;155:3538-49.

[11] Hosoyama T, Nishijo K, Garcia MM, Schaffer BS, Ohshima-Hosoyama S, Prajapati SI et al. A postnatal Pax7 progenitor gives rise to pituitary adenomas. Genes Cancer 2010;1:388-402.

[12] Fauquier T, Rizzoti K, Dattani M, Lovell-Badge R, Robinson IC. SOX2-expressing progenitor cells generate all of the major cell types in the adult mouse pituitary gland. Proc Natl Acad Sci USA 2008;105:2907-12.

[13] Kelberman D, Rizzoti K, Ramani VAC, Bitner-Glindzicz M, Cianfarani S, Collins J et al. Mutations within Sox2/Sox2 are associated with abnormalities in the hypothalamo-pituitary-gonadal axis in mice and humans. J Clin Invest 2006;116:2442-55.

[14] Andoniadou CL, Matsushima D, Mousavy Gharavy SN, Signore M, Mackintosh AI, Schaeffer M. Stem/Progenitor cells in the adult mouse pituitary support organ homeostasis and have tumor inducing potential. Cell Stem Cell 2013;13:433-45.

[15] Hanson JM, Teske E, Voorhout G, Galac S, Kooistra HS, Meij BP. Prognostic factors for outcome after transsphenoidal hypophysectomy in dogs with pituitary-dependent hyperadrenocorticism. J Neurosurg 2007;107:830-40.

[16] Galac S, Kooistra HS, Teske E, Rijnberk A. Urinary corticoid/creatinine ratios in the differentiation between pituitary-dependent hyperadrenocorticism and hyperadrenocorticism due to adrenocortical tumour in the dog. Vet Q 1997;19:17-20.

[17] Meij B, Voorhout G, Rijnberk A. Progress in transsphenoidal hypophysectomy for treatment of pituitary-dependent hyperadrenocorticism in dogs and cats. Molecular and cellular endocrinology 2002;197:89-96.

[18] van Rijn SJ, Riemers FM, van den Heuvel D, Wolfswinkel J, Hofland L, Meij BP et al. Expression stability of reference genes for quantitative RT-PCR of healthy and diseased pituitary tissue samples varies between humans, mice, and dogs. Molecular neurobiology 2014;49:893-9.

[19] Kooistra HS, Galac S. Recent advances in the diagnosis of Cushing's syndrome in dogs. Top Companion Anim Med 2012;27:21-4.

[20] Peterson ME, Krieger DT, Drucker WD, Halmi NS. Immunocytochemical study of the hypophysis in 25 dogs with pituitary-dependent hyperadrenocorticism. Acta Endocrinol 1982;101:15-24.

[21] Mbikay M, Seidah NG, Chrétien M. Neuroendocrine secretory protein 7B2: structure, expression and functions. Biochem J 2001;357:329-42.

[22] Miller R, Aaron W, Toneff T, Vishnuvardhan D, Beinfeld MC, Hook VY. Obliteration of α-melanocyte-stimulating hormone derived from POMC in pituitary and brains of PC2-deficient mice. Journal of neurochemistry 2003;86:556-63.

[23] Moore HP, Andresen JM, Eaton BA, Grabe M, Haugwitz M, Wu MM et al. Biosynthesis and secretion of pituitary hormones: dynamics and regulation. Arch Physiol Biochem 2002;110:16-25.

[24] Briassoulis G, Damjanovic S, Xekouki P, Lefebvre H, Stratakis CA. The glucocorticoid receptor and its expression in the anterior pituitary and the adrenal cortex: a source of variation in hypothalamic-pituitary-adrenal axis function; implications for pituitary and adrenal tumors. Endocr Pract 2011;17:941-8.

[25] Alatzoglou KS, Kelberman D, Dattani MT. The role of SOX proteins in normal pituitary developement. J Endocrinol 2009;200:245-58.

[26] Rizzoti K, Akiyama H, Lovell-Badge R. Mobilized adult pituitary stem cells contribute to endocrine regeneration in response to physiological demand. Cell Stem Cell 2013;13:419-32.

8. Discussion and conclusion

Cushing's disease, or pituitary-dependent hypercortisolism (PDH), is a common endocrinopathy in dogs and is caused by an ACTH-producing pituitary tumor. As described in chapter 3, several proliferative markers have been investigated in the canine pituitary adenoma. However, research into the pathogenesis of canine PDH is limited, and no markers are found yet that may support in prognosis or that can be used as therapeutic targets. It is believed that tumors exist of two types of cells, tumorigenic and non-tumorigenic cells. Tumorigenic cells, or CSCs, are responsible for growth, maintenance and progression of tumors, and give rise to the non-tumorigenic cells [5,6]. It has been shown that it is possible to sort SP cells from mice [7] and canine [3] healthy pituitaries by means of FACS analysis, and these SP cells showed stem cell characteristics. In the present study, the protocol for the isolation of these stem cells was investigated, with the goal to isolate high quality RNA from the sorted stem cells, suitable for gene expression analysis. Next to the optimization of the FACS technique, the expression of the transcription factor Pax7 and its related genes was investigated.

First, the FACS technique was optimized, in order to sort SP cells from the healthy pituitary and pituitary adenoma. The SP proportion is influenced by cell density during staining, staining volume and percentage life cells [9]. As described in chapter 5, SP appearance differed among the samples, as well as the proportion SP cells. The gating of the SP is an amendable technique and it is important that all samples are treated the same. However, the work with patient material, different DNA content of the cells and high variations in cell viability, made it not possible to use the same gating in all samples to isolate SP cells.

High RNA yields and quality start with viable cells and a high total life cell count in the samples, prior to FACS analysis. In the present study, two different cell digestion protocols were used. All adenoma samples (except one) were digested by collagenase, while all healthy pituitaries (except one) were digested by trypsinization, as described previously [3,10]. In both protocols, two different kinds of media were used. To investigate with which protocol the highest percentage life cells could be obtained, we tested different media, FACS machines, collections methods and fresh/frozen samples. All adenoma samples were frozen and stored for 1 to 4 years, while healthy pituitaries were frozen or freshly processed. High variations in percentage life cells was found among the samples and ranged from 2 to 44%. Interestingly, an increased percentage life cells was found in the frozen samples. This may be due to a change in media, that occurred almost simultaneously with the use of fresh instead of frozen samples. Also, all fresh samples were sorted on a BD Influx, and all frozen samples were sorted on the FACSAria II (SORP). Since several parameters were changed in the same time, it was hard to explain how cell survival is influenced.

Second, the best method to collect cells after FACS analysis, was investigated. To obtain high quality RNA, several collection methods were used. Sorting bone marrow cells, collection in RLT-buffer with β-ME gave the best results (RIN 8.6). Moreover, collection in empty tubes or in 3 ml RNA *Later®* gave satisfying results as well (RIN >6.5). RNA extracted of sorted pituitary gland cells did not give the same RIN values, but RNA was much more degraded. Differences in cell viability and with that apoptosis during FACS analysis, may have contributed to the poor RNA quality of the samples.

Contrary reports were published about the importance of RNA quality and reliability of gene expression analysis. Some report changes in gene expression in degraded samples [11,12], while

others report only small alterations in expression, and in only certain genes [13]. In the present study, samples were sorted using two different FACS machines and gene expression analysis of three reference genes (*tbp, hmbs, ywahz*) was performed. Although the mean Ct of the frozen samples sorted on the FACSAria II (SORP) was increased with 2.5 threshold cycles compared with the mean Ct of fresh samples sorted on the BD Influx, the difference was not significant. However, by far not in all samples sorted on the FACSaria II (SORP), expression of the reference genes could be measured, whereas in all samples (except one) sorted on the BD Influx, expression of all three reference genes was measured. This indicates for a more reliable gene expression in the samples sorted with the BD Influx. However, it was not possible to determine whether this finding was due to the type of FACS machine, or due to freezing of the samples.

The last research question studied was based on the Pax7 results of van Rijn *et al* (unpublished). Pax7 protein expression was found in about 34% of the canine pituitary adenomas, and in one out of six healthy pituitaries. These findings were in contrary with the results Budry *et al* [4] presented, were it was concluded that Pax7 expression was restricted to the melanotrope cells of the intermediate lobe. To further investigate the proportion of melanotrope cells in pituitary adenomas, PC2 protein expression was determined, but no positive cells were found in neither healthy pituitary nor pituitary adenomas. Besides, Pax7 expression was down regulated in the pituitary adenoma samples, while it was hypothesized that Pax7 immunopositivity was linked to an intermediate lobe origin of the pituitary adenoma. Also, PC2, Drd2 and Sox2 were significantly down regulated in the pituitary adenomas, which is in contrary to other reports. However, in this study canine pituitary tissue was used, whereas most research is done in mice, which makes it challenging to compare the results.

Concluding from the first two chapters of this study (chapter 5 & 6), it is possible to sort SP cells from the canine healthy pituitaries and pituitary adenomas. SP appearance and proportion differed among the samples. To collect the SP cells after sorting, different collection methods were tested. From bone marrow samples, high quality RNA could be isolated, which was not possible in the pituitary gland samples. Differences in cell viability, susceptibility to the forces during FACS and freezing of the samples may induce cell damage and with that, RNA degradation. However, it was possible to analyze gene expression in the sorted samples, despite low RIN values. To compare gene expression in the sorted samples with low RIN values among each other, the influence of RNA degradation on gene expression in pituitary gland samples has to be investigated.

In chapter 7, Pax7 expression was further investigated. The absence of PC2 expression and the down regulation of Pax7, PC2, Sox2 and Drd2 in pituitary adenomas, evoked new research questions, instead of giving an explanation for the Pax7 results found by van Rijn *et al* (unpublished). To explain these results, further research has to be done in protein expression of the investigated genes. Besides, other genes related to Pax7, PC2 and the ACTH secretion, like 7B2 or PC1, have to be investigated.

8.1 Cited references

[1] Magee JA, Piskounova E, Morrison SJ. Cancer stem cells: impact, heterogeneity, and uncertainty. Cancer Cell 2012;21:283-96.

[2] Meacham CE, Morrison SJ. Tumour heterogeneity and cancer cell plasticity. Nature 2013;501:328-37.

[3] Chen J, Gremeaux L, Fu Q, Liekens D, Van Laere S, Vankelecom H. Pituitary progenitor cells tracked down by side population dissection. Stem Cells 2009;27:1182-95.

[4] van Rijn SJ, Gremeaux L, Riemers FM, Brinkhof B, Vankelecom H, Penning LC et al. Identification and characterisation of side population cells in the canine pituitary gland. Veterinary Journal 2012;192:476-82.

[5] Mo SL, Li J, Loh YS, Brown RD, Smith AL, Chen Y et al. Factors influencing the abundance of the side population in a human myeloma cell line. Bone Marrow Res 2011;Epub.

[6] Denef C, Maertens P, Allaerts W, Mignon A, Robberecht W, Swennen L et al. Cell-to-cell communication in peptide target cells of anterior pituitary. Methods in enzymology 1989;168:47-71.

[7] Birdsill AC, Walker DG, Lue L, Sue LI, Beach TG. Postmortem interval effect on RNA and gene expression in human brain tissue. Cell Tissue Bank 2011;12:311-8.

[8] Koppelkamm A, Vennemann B, Lutz-Bonengel S, F, T, Vennemann M. RNA integrity in post-mortem samples: influencing parameters and implications on RT-qPCR assays. Int J Legal Med 2011;125:573-80.

[9] Opitz L, Salinas-Riester G, Grade M, Jung K, Jo P, Emons G et al. Impact of RNA degradation on gene expression profiling. BMC Med Genomics 2010;3.

[10] Budry L, Balsalobre A, Gauthier Y, Khetchoumian K, L'honoré A, Vallette S et al. The selector gene Pax7 dictates alternate pituitary cell fates through its pioneer action on chromatin remodeling. Genes & development 2012;26:2299-310.

9. List of abbreviations

α-MSH	alpha-Melanocyte-stimulating hormone
ABC	ATP-binding cassette
ABCG2	ATP-binding cassette sub-family G member 2
ACTH	Adrenocorticotrophic hormone
AL	Anterior lobe
ATP	Adenosine triphosphate
AVP	Arginine vasopressin
Axin2	axis inhibition protein 2
β-ME	β-Mercaptoethanol
Bmi-1	Polycomb ring finger oncogene
CD34	Cluster of differentiation molecule 34
CD133	Cluster of differentiation molecule 133
CC	Correlation coefficient
CDI	Diabetes insipidus
CHO	Chinese hamster ovary
CNS	Central nervous system
CSC	Cancer stem cell
CT	Computed tomography
Ct	Threshold cycle
CRH	Corticotropin releasing hormone
DAB	3,3'-diaminobenzidine
dCT	Delta cycle threshold
DMSO	Dimethylsulfoxide
Drd2	Dopamine receptor D2
EGFR	Epidermal growth factor receptor
ER	Endoplasmatic reticulum
FACS	Fluorescence activated cell sorting
FCS	Foetal Calve Serum
FSH	Follicle-stimulating hormone
GC	Glucocorticoid
GH	Growth hormone
hmbs	hydroxymethylbilane synthase
HPA	Hypothalamus-pituitary-adrenal
IL	Intermediate lobe
LH	Luteinizing hormone
Lhx3	LIM class homeodomain transcription factor 3
LIF	Leukemic inhibitory factor
MCM	Minichromosome maintenance
MCM7	Minichromosome maintenance 7
MCM2	Minichromosome maintenance 2
MP	Main population
MRI	Magnetic resonance imaging
NGS	Normal goat serum

o,p'-DDD	1-chloro-2-[2,2-dichloro-1-(4-chlorophenyl)-ethyl]-benzene
Pax7	Paired box 7
P/B	Pituitary/brain
PBS	Phosphate buffered saline
PC1	Prohormone convertase 1
PC2	Prohormone convertase 2
PCNA	Proliferating cell nuclear antigen
PDH	Pituitary-dependent hypercortisolism
pg	Picogram
P-gp	Phosphorylated p-glycoprotein
PI	Propridium Iodine
PL	Posterior lobe
PNS	Peripheral nervous system
POMC	Proopiomelanocortin
PMI	Post-mortem interval
PRL	Prolactin
PSI	Pounds per square inch
qPCR	Quantitative polymerase chain reaction
RBC	Red blood cell
RIN	RNA Integrity Number
RP	Rathke's pounch
RT	Room temperature
Shh	Sonic hedgehog
Sox2	SRY (sex determining region Y)-box 2
SP	Side population
tbp	Tata box binding protein
TBST	Tris-buffered saline +0,1%Tween
Thmbs2	thrombospondin 2
THP1	Human acute monocytic leukemia cell line
Tpit	Pituitary T-box transcription factor
TKI	Tyrosin kinase inhibitor
TSH	Thyroid-stimulating hormone
UCCR	Urinary corticoid:creatinine ratio
ywahz	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide

10. Orientation

During my research minor I orientated in the field of veterinary research by following courses and attending congresses/seminars. Besides, I practiced my scientific writing by submitting two grant proposals.

Courses

29 September – 3 October	Radiology course, Faculty of Veterinary Medicine, Utrecht University
15 – 26 September	Molecular Translational Oncology, Radboud University, Nijmegen

Congresses

17 April 2014	European Veterinary Conference 'de Voorjaarsdagen', Amsterdam
9 May 2014	Dutch Stem Cell Meeting, Groningen

Grant proposals

October	Morris Animal Foundation (MAF), Veterinary Student Scholars
June	National Canine Cancer Foundation, Letter of intent

11. Dankwoord

Mijn enthousiasme voor het onderzoek kwam niet alleen door de goede onderzoeksresultaten, maar toch ook vooral door iedereen met wie ik heb samengewerkt het afgelopen jaar. Er zijn dus een heleboel mensen die ik moet bedanken.

Om aan het begin van een doorsnee labdag te beginnen: de 'koffieleuten' die ervoor zorgde dat 's ochtends om 08:00 uur de koffie klaar stond. Niets beters dan verse koffie, en soms een prikkelde vraag om de dag mee te beginnen (Ellie: 'weet jij nog wie Loekie de Leeuw is?').

Elpetra, Frank, Saskia en Alberta voor het inwerken in de primaire kast, qPCR, immunohistochemistry en RNA isolatie. Het is altijd spannend om voor de eerste keer een van de technieken zelf uit te voeren, maar door de goede inwerking lukte het prima.

Iedereen die met mij mee naar beneden is gelopen, om samples naar het stikstof te brengen of om op te halen. Bas, Manon, Frank, Frances, Arend en Jeanette. Arend vond het om 07:30 's ochtends zelfs geen probleem, al geen de meeste credits toch echt naar Jeanette, die is zo vaak meegegaan….

De orthopedie groep. Wat een fantastische groep om mee samen te werken! Iedereen helpt elkaar, er worden cadeautjes geregeld bij promoties, elke dinsdag hardgelopen, samen pannenkoeken eten, BBQ-en… Frances, Nicole, Alberto, Michelle, Anna, Marianna, Björn, Annemieke, Lucy, Eyleen, Margot, Ferdi, Nadi, Frank, Jeanette, Saskia, Nicoline, Leili en Bianca, bedankt!

Oké, Nicole krijgt nog een extra bedankje. Dankzij jou ben ik begonnen met hardlopen, en wil ik niet meer stoppen…

Ook buiten het lab zijn er veel mensen die mij hebben geholpen. Harrie, bedankt voor het verzamelen van de hypofyse. Als je er een keer niet was, wisten we meteen wat we misten. Guy, bedankt voor de hulp bij het interpreteren van de PC2 kleuring. Vaak kon ik snel bij je terecht met mijn vragen, zodat ik weer verder kon met de experimenten. Ger en Stefan, bedankt voor het FACSen van mijn cellen. Met Stefan heb ik vele discussies gevoerd en verschenen er vaak interessante publicaties in mijn mailbox, nog voordat ik zelf terug was op het lab. Daar hadden we het dan over gehad tijdens het FACSen.

Nadi, bedankt voor je input de afgelopen 2 maanden. We hebben samen in het lab gewerkt en nagedacht hoe we onze cell counts beter kunnen krijgen. Ik ben ontzettend benieuwd hoe het afloopt in Leuven, maar met jouw inzet moet het goed komen!

Louis, je hebt vaak mijn stoel en pen 'in bruikleen genomen', maar voorruit. In ruil voor al jouw input en nieuwe ideeën voor mijn onderzoek, vond ik dat prima. En, de vrijdagmiddag biertjes smaakten prima!

Björn, hoe een praatje met mijn vader tijdens de Najaarsdagen 2 (of 3?) jaar geleden kan eindigen met 8 maanden hypofyse onderzoek. We hebben vaak samen in de OK gestaan, waar met uiterste precisie de hypofyse uit de kat/hond werd verwijderd. Ik heb alles over de procedure kunnen vragen, en je hebt voor vele adenomen gezorgd, die ik vervolgens weer kon onderzoeken.

Marianna, jij weet van de orthopedie groep een echte groep te maken. Samen eten en samen rennen zorgt ervoor dat iedereen ook goed samenwerkt. Ik heb echt een toptijd gehad in het lab, en dat kwam mede door alles wat we er naast deden. Daarnaast moet ik je bedanken voor alle oplossingen en nieuwe ideeën die je had, om het hypofyse-cellen-mysterie op te lossen.

Last, but not least (not at all), Sarah. Bedankt voor al je input. Voor de snelheid waarmee je mijn verslag had nagekeken (ik ben nog steeds verbaast) en voor de vrijheid die je me gaf in de uitvoering van het onderzoek. Ik hoop echt dat we binnenkort topkwaliteit RNA isoleren! Maar tot die tijd, alle aandacht naar je promotie. Maar dat gaat zeker lukken☺.

Appendix A: Protocols for cell dispersion and Hoechst staining

A1. Introduction

To establish the technique of FACS analysis of pituitary tissue at the Hubrecht laboratory (Utrecht), canine bone marrow cells where used to adjust the right settings for a cell sort with/without Hoechst 33342 staining. Once SP cells were successfully isolated from bone marrow cells, healthy pituitary- and adenoma samples where sorted.

A2. Media

- 20x Erytrhocytenlysis-buffer (20x RBC)
    - For 1 liter:
    - 165,8 g NH_4CL(3,1 M)
    - 20,0 g $KHCO_3$ (0,2 M)
    - 7,4 g $Na_2EDTA.2H_2O$
    - solve in 800 ml MilliQ
    - adjust to pH 7,4 with NaOH
    - fill up to 1 l with MilliQ
    - Autoclave
- Enriched D-MEM solution
    - 100 ml D-MEM (4500 mg/L glucose, L-glutamine, pyruvate 110 mg/L, GIBCO, Invitrogen, Breda, the Netherlands)
    - 1 ml Penicillin Streptomycin (5000:5000, GIBCO, Invitrogen)
    - 0,6 g Hepes (Sigma-Aldrich, Zwijndrecht, the Netherlands)
    - 0,3% Bovine Serum Albumin (BSA, Sigma-Aldrich)
- Enriched HBSS solution
    - 100 ml HBSS (PAA, Pasching, Austria)
    - 0,476 g Hepes (Sigma-Aldrich)
    - 1 ml Penicillin Streptomycin
    - 0,3 g Bovine Serum Albumin (BSA)(Sigma-Aldrich)
- EDTA solution
    - 100 ml enriched HBSS solution
    - 74,4 mg EDTA (Sigma-Aldrich)
- Trypsin solution
    - 2,5w/v% in enriched D-MEM solution (±500 U/ml)
    - Warm at 37°C to dissolve
- Collagenase solution
    - 5 ml α-MEM (GIBCO, Invitrogen)
    - 5 mg Collagenase IV (Sigma-Aldrich)
- DNase solution
    - 2 ml enriched D-MEM solution
    - 20 µl DNase (Sigma-Aldrich)
- Serum-free defined culture medium (1A)
    - 100 ml DMEM 1;1/F12 (GIBCO, Invitrogen)
    - 0,5 mg Transferrin (Bovine, GIBCO, Invitrogen)

    - 0,5 mg Insulin (Sigma-Aldrich)
    - 1 ml Penicillin Streptomycin
    - 60 µl absolut ethanol (Merck, Germany),
    - 0,1 mg catalase (Sigma-Aldrich)
    - 0,1 g $NaHCO_3$ (Merck, Germany),
    - 0,5 g BSA
- Serum-free defined culture medium (2A)
    - DMEM-glutamax
    - 2% Foetal Calf Serum (FCS) (PAA Pasching, Austria)
    - 10 mM Hepes
    - 2.5 µg/ml Insulin
- Sorting solution (1B)
    - HBSS (GIBCO)
    - 2% FCS
    - PI 1:5000
    - 0,4% DNase
    - 0,025% EDTA
- Sorting solution (2B)
    - HBSS
    - 2% FCS
    - 2 µg/ml PI

All media are filtered through a 22µm filter.

A3. Materials and protocols

A3.1. Freezing of healthy pituitary and adenoma samples

- Make medium: Leibowitz (GIBCO) + 10% FCS
- Add 1,5 ml per cryovial; store at -70°C until use
- Before use, thaw cryovial
- Collect tissue in a petridish and cut into small pieces (2x2 mm) using 2 surgical blades nr. 11
- Drop 4-5 pieces of tissue in a cryovial
- Keep the vials on ice
- Add 10% DMSO to each vial
- Leave the vials at room temperature (RT) for 5 minutes, so the DMSO can infiltrate the tissue pieces
- Freeze gradually: put vials in Mr. Frosty (which contains isopropanol) and put in -80°C
- After 12-24 hours, transfer to liquid nitrogen (-200°C)

A3.2. Cell dispersion of bone marrow

- The dog is euthanized
- Before collecting the samples, write down present date, GDL number, identification number, gender and birth date of the dog
- The fore- or hind limbs are dissected. Two legs will give a right amount of bone marrow cells

- The legs are washed twice with Hibiscrub® to remove loose hairs, followed by two washes with Hibisol®
- Remove tissue and muscles with a surgical blade nr. 10 and dissect the femur (hind limb) or humerus (forelimb) free
- Saw the bone with an electrical saw on both sides in the metaphysis
- Collect the bones into prewarmed α-MEM (GIBCO) + heparine (LEO Pharma, Ballerup, Denmark) + 2%FCS + 1% pen-strep (GIBCO)
- Use a Needle holder (Mayo-Hegar) to excavate the bones
- Flush the bones with α-MEM + heparine + 2%FCS + 1% pen-strep (GIBCO) and collect the cells
- Centrifuge cell suspension (5 min 1500 rpm)
- Remove the upper layer which contains fat
- Resuspend cells
- Store at RT
- Make RBC lysis buffer. Filter to sterilize (0,22µm)
- RBC lysis 1:4
- Mix quietly by hand
- Place horizontal (10 min; RT)
- Centrifuge cell suspension (5 min; 1500 rpm; RT)
- Wash twice with hanks
- Filter cell suspension (70µm filter)
- Centrifuge cell suspension (5 min; 1500 rpm; RT)
- Resuspend cells in prewarmed DMEM + 2%FCS +10 mM Hepes (Sigma-Aldrich) +1% pen-strep
- Count cells

A3.3. Cell dispersion of healthy pituitary

- Thaw sample in a water bath at 37°C
- Transfer tissue to a 50 ml tube
- Was twice with medium A
    - Make trypsin solution
- Remove supernatant
- Transfer the tissue to a petridish and add 3 ml of medium A
- cut into small blocks with a surgical blade nr. 11
- Transfer fragments to a 50 ml tube, rinse petridish with medium A and add to the tube
- Add 2 ml of trypsin solution
- Incubation in a shaking water bath (15 min; 37°C; 60 rpm)
    - Make DNase solution
    - Make 3 ml medium A + 3% BSA
- Do not remove supernatant
- Add 2 ml of DNase solution
- Gentle move to mix and disperse cell clumps
- Wait 1 min

- After fragments are sedimented to the bottom, remove the supernatant
- Add 2 ml of B1 medium
- Incubate(5 min; 37°C)
- After fragments sedimented to the bottom, remove supernatant
- Wash with 5 ml medium C +10% FCS
- Wash 2 times with 5 ml medium C
- Remove supernatant
- Add 0.75 ml medium C and transfer to Wheaton potter and pastel
- Dispersion met Wheaton potter and pastel
- Disperse cells and add supernatant (with dispersed cells) to a tube containing 4.5 ml medium A + 7.4µl DNase
- Add 0.75 ml of medium C to the undissociated tissue blocks and disperse again. Repart maximally 5-7 times.
- The obtained cell suspension is filtered through a nylon mesh (70 µm) in a 50 ml tube
- Add 3 ml of 3% BSA layer at the bottom of the tube
- Centrifuge (5 min;1500 rpm; RT)
- Resuspend the cell pellet in 1 ml serum-free defined culture medium (1A or 2A)
- Count cells

A3.4. Cell dispersion of pituitary adenoma

- Thaw sample in a water bath at 37°C
- Was twice with medium A
    - Make Collagenase IV solution (5ml; 1mg/ml in α-MEM) filter (0,22nm) to sterilize
- Remove supernatant
- Transfer the tissue to a petridish and add 3 ml of medium A
- cut into small blocks with a surgical blade nr. 11
- Transfer fragments to a 50 ml tube, rinse petridish with medium A and add to the tube
- Remove supernatant
- Add 5 ml collagenase IV solution
- Incubate (37°C; 90 min)
    - Make PBS + 20% FCS
- Pipette up and down every 20 minutes
- Filter through a 40 nm nylon mesh in a 10 ml tube
- Transfer filtrate to 10 ml tube (rinse with PBS+20%FBS)
- Disperse remaining pieces with a pipet with PBS+20%FBS
- Filter these remaining cells (and add further PBS+20%FBS till α-MEM 2-fold diluted, to inactivate collagenase)
- Centrifuge (5 min; 1500 rpm; RT)
- Resuspend pellet in 5 ml PBS+20%FBS
- Centrifuge (5 min; 1500 rpm; RT)
- Resuspend pellet in 1 ml synthetic medium (1B or 2B)
- Count cells

A3.5. Hoechst staining

- Dilute to 1 x10^6 cells/ml in
    - Bone marrow: prewarmed DMEM + 2%FCS +10 mM Hepes +1% pen-strep (Gibco)
    - Pituitary/adenoma: serum-free defined culture medium (1A or 2A)
- Split the samples
    - Bone marrow control sample: 1 ml
    - Bone marrow sample: 5-8 ml
    - Pituitary/adenoma control sample: max 1 ml
    - Pituitary/adenoma sample: >1 ml
- Add Verapamil (Centrafarm) to the control sample
    - Bone marrow: 50µM
    - Pituitary/adenoma: 100 µM
- Incubate (20 min; 37°C)
- Add Hoechst 33342 (Sigma-Aldrich) in both samples
    - Bone marrow: 5 µg/ml
    - Pituitary/adenoma: 2.5 µg/ml
- Incubate (90 min; 37°C)
- Gently mix after 45 minutes
- After incubation cool cells rapidly on ice
- Dilute 1:1 with ice cold serum-free defined culture medium (1A or 2A)
- Centrifuge(5 min; 1500 rpm; 4°C)
- keep on ice
- Resuspend cells in sorting solution (1B or 2B)
    - Bone marrow control sample: 1 x10^6 cells/ml
    - Bone marrow sample: 5-8 x10^6 cells/ml
    - Pituitary/adenoma control sample: max 1 x10^6 cells/ml
    - Pituitary/adenoma sample: >1 x10^6 cells/ml
- Pass through a 40 πm mesh to remove cell debris
- FACS

Appendix B: Protocol for immunohistochemistry staining

B1. Introduction

For the optimization of the PC2 staining protocol, different methods of antigen retrieval were tested. The reagents used for the optimization and the final protocol, are listed in section B2. The protocol used for the staining, is depicted in section B3.

B2. Reagents

- Tris-Buffered Saline (TBS) 10x
    - 1.21 g Tris Base (Merck, Darmstadt, Germany)
    - 9 g NaCl (Merck, Darmstadt, Germany)
    - solve in 1000 ml milliQ
    - Adjust pH 9.0
- TBS+0.1%Tween20
    - 100 ml TBS 10x
    - 900 ml milliQ
    - 1 ml Tween20 (Boom B.V., Meppel, the Netherlands)
- Phosphate-Buffered Saline (PBS) 10x
    - 80 g NaCl
    - 2 g KCL (Sigma-Aldrich, St. Louis, USA)
    - 14.4 g Na2HPO4 (Merck KGaA, Darmstadt, Germany)
    - 2.4 KH2PO4 (Merck, Darmstadt, Germany)
    - Solve in 1000 ml milliQ
    - Adjust pH 7,4
- PBS+0.1% Tween20
    - 100 ml PBS 10x
    - 900 ml milliQ
    - 1 ml Tween20
- CIT buffer (10 nM Citrate buffer)
    - 2.1 g Citric acid (Merck, Darmstadt, Germany)
    - Solve in 1000 ml milliQ
    - Adjust pH 9.0
- Tris-EDTA buffer
    - 1.21 g Tris Base
    - 0.37 g EDTA (Merck, Darmstadt, Germany)
    - Solve in 1000 ml milliQ
    - Adjust pH 9.0
- Pronase solution 0.05%
    - 0.005 g pronase (Roche Diagnostics GmbH, Mannheim, Germany)
    - Solve in 10 ml PBS

B3.1 PC2 immunohistochemistry

Protocol:

- Dehydrate paraffin sections in:
  - Xylene I — 5 min
  - Xylene II — 5 min
  - 96% EtOH — 5 min
  - 80% EtOH — 5 min
  - 70% EtOH — 5 min
  - 60% EtOH — 5 min
  - TBS — 1 min
- Perform antigen retrieval
  - Citrate in water bath — 15 min; 98°C
  - EDTA buffer in water bath — 20 min; 98°C
  - 100 µl Pronase solution(0.05%) — 6 min ; 37°C
  - Proteinase-K — 6 min; 37°C
  - Proteinase-K — 3 min; 37°C
- Leave slides to cool down
  - Citrate /tris-EDTA buffer — 60 min; RT
  - Pronase / Proteinase-K — 10 min; RT

  Rinse 2 times in TBST0.1% — 5 min
- Make circles around tissue with Pap-pen
- Keep tissue from drying out, TBS on slides
- Block with Endogenous enzyme block (Dako S2003), 2-3drops — 10 min; RT
- Rinse 2 times with TBST0.1% — 5 min
- Block in 10% normal goat serum, 100µl — 60 min; RT
- Don't rinse slides, but touch carefully dry
- Incubate slides with primary antibody diluted in antibody diluent,100 µl/slide — (O/N; 4˚C)

Continuation next day:

- Rinse 3 times with TBST0.1% — 5 min
- Incubate slides with Secondary antibody 2-3 drops — 30 min; RT
- Rinse 3 times with TBST0.1% — 5 min
- Incubate slides with DAB (100µl/slide) — 10 min; RT
- Rinse 3 times with TBST0.1% — 5 min
- Counterstain with haematoxylin — 10-30sec
- Rinse with running Tap-water — 5-10 min
- Hydrate paraffin sections in:
  - 60% EtOH — 5 min
  - 70% EtOH — 5 min
  - 80% EtOH — 5 min
  - 96% EtOH — 5 min
  - 96% EtOH — 5 min
  - 100% EtOH — 5 min

- Xylene II 5 min
- Xylene I 5 min

- Coverslip with permanent mounting medium

Appendix C: Course information and grade

Molecular and Translational Oncology

Course ID: NWI-BM043B
Radboud University, Nijmegen
September 16 – 27, 2013

12 hrs lecture
8 hrs problem session
50 hrs individual study period

Pre-requisites

Biochemistry and Molecular Biology II, Celbiologie van dieren and Functional Genomics. This prior knowledge can be found in Lodish 6th edition, Chapters 4, 6, 7, 8, 15, 16, 20, 21, 24.

Objectives

- The student acquires knowledge and insight regarding the molecular mechanisms that underlie cancer development.
- The student acquires insight and understanding on how molecular knowledge can be translated into clinical practice.
- The student can identify the overlaps as well as differences between cancer related topics
- The student is able to use the acquired knowledge to identify and formulate a cancer related (molecular) problem and knows how this problem molecularly could be approached

Contents

This course aims to showcase current insights in the molecular mechanisms that lead to cancer and how these can be translated into clinical practice. Special emphasis will be on epigenetics (heritable modifications of chromosomes), transcription factors, signaling pathways and the molecular biology of tumor suppressors in the context of leukemia, colorectal cancer, prostate cancer and immunotherapy.

Subjects

- Hallmarks of cancer
- The epigenetics of leukemia and colorectal cancer
- Ubiquitylation in normal and malignant hematopoiesis
- Implementation of innovative basic science in clinical practice
- Immunotherapy of cancer
- Genetics and biology of pediatric acute lymphoblastic leukemia
- Role of oncogenic tyrosine kinases in cancer
- Molecular mechanism underlying prostate cancer

M.G. Pouwer
Nedereindsestraat 33 B
4041 XE KESTEREN

Betreft Studievoortgangsoverzicht 4514904
Datum 14 oktober 2014
Opleiding M Medical Biology voltijd **Cohort** 2014
Examenprogramma NWI-MA-MB-14 - Master Medical Biology

European Credits

Examenprogramma	Minimum te behalen punten	Behaalde punten	Voldaan
Basisprogramma	120,0	0,0	Nee
Overig		3,0	
Totaal	**120,0**	**3,0**	**Nee**

Programma

Examenonderdeel	Type	Minimum te behalen punten	Behaalde punten	Voldaan
alle onderstaande onderdelen				
1. Mastertrack	Specialisatie	120,0	0,0	Nee
2. Extra-curriculaire vakken	Keuze	0,0	0,0	Ja
Totaal		**120,0**	**0,0**	**Nee**

Resultaten - Overig (deze resultaten tellen niet mee voor dit examenprogramma)

Cursus			Punten	Datum	Resultaat
NWI-BM043B	Molecular and Translational Oncology		3,0	26-09-2014	7,0
	Tentamen	*weging 1*		*26-09-2014*	*7*
NWI-BM054C	Master Portfolio Biosciences		(0,0)		

Aan deze gegevens kunnen geen rechten worden ontleend.

Appendix D: Data of cell count and qPCR

D1. Introduction

In total, 46 samples were digested. Patient information, protocol, cell count, SP proportion and RNA yields are depicted in table D1. Quantitative PCR was performed on a subset of the sorted samples, as depicted in table D2. In table D3, Ct values of the qPCR described in chapter 7 are listed, including calculations of the mean M-fold using the Livak and Pfaffl method.

Sample nr.	date FACS	sample	Species	sample nr	Date of sample collection	Owner	Protocol	Total cell count	Total life count	% life	Media
1	14-3-2014	adenoom	Canine	910051	2-2-2010		Collagenase	1,42x10^6	370000/ml	26	1A/1B
2	14-3-2014	adenoom	Canine	1311796	6-1-2014		Collagenase	15x10^6	6,2x10^6	41	1A/1B
3	25-4-2014	hypofsye	Canine	7199261	2-4-2014	GDL	Trypsine	1,46x10^6	455.000	31	1A/1B
4	25-4-2014	hypofyse	Canine	104577	25-3-2014	GDL	Trypsine	1,07x10^6	252.000	24	1A/1B
5	1-5-2014	hypofyse	Canine	104664	30-4-2014	GDL	Collagenase	126.000	30.300	24	1A/1B
6	12-5-2014	Adenoom	Canine	1403425	6-5-2014	Dayo/Thijssen	Collagenase	6,76x10^6	1,04x10^6	15	1A/1B
7	12-5-2014	hypofyse	Canine	1037341	7-5-2014	GDL	Trypsine	1,74x10^6	482.700	28	1A/1B
8	28-5-2014	hypofyse	Canine	843181	22-4-2014	GDL	Trypsine	1,16x10^6	248.000	21	1A/1B
9	28-5-2014	hypofyse	Canine	865575	22-4-2014	GDL	Trypsine	278.000	80.900	29	1A/1B
10	28-5-2014	hypofyse	Canine	853968	22-4-2014	GDL	Trypsine	1,17x10^6	167.000	14	1A/1B
11	28-5-2014	hypofyse	Canine	879835	22-4-2014	GDL	Trypsine	789.000	177.000	22	1A/1B
12	4-6-2014	adenoom	Canine	1004168	1-6-2010	Di Santo/Dafne	Collagenase	2,78x10^6	814.000	29	1A/1B
13	4-6-2014	adenoom	Canine	908650	8-6-2010	Poppel/Lapje	Collagenase	6,48x10^6	1,2x10^6	19	1A/1B
14	4-6-2014	adenoom	Canine	1301722	5-3-2013	Mazaheri/Lucy	Collagenase	2,02x10^6	566.000	28	1A/1B
15	4-6-2014	adenoom	Canine	1301566	4-3-2013	Agthoven/Bintang	Collagenase	1,08x10^7	4,22x10^6	39	1A/1B
16	4-6-2014	adenoom	Canine	1401318	11-3-2014	Staats/Dorus	Collagenase	708.000	46.000	21	1A/1B
17	4-6-2014	adenoom	Canine	909609	9-2-2010	Wagenaar/Tina	Collagenase	1,8x10^6	460.000	26	1A/1B
18	4-6-2014	adenoom	Canine	1404636	27-5-2014	Sep/Thomasino	Collagenase	4,14x10^6	971.000	23	1A/1B
19	4-6-2014	adenoom	Canine	1000706	9-3-2010	Abeelen/Kai	Collagenase	1,95x10^6	642.000	33	1A/1B
20	11-6-2014	adenoom	Canine	1308566	1-10-2013	van Es/Sjors	Collagenase	172.000	10.100	6	1A/1B
21	11-6-2014	adenoom	Canine	1311131	11-11-2013	Verheule/Reno	Collagenase	1,66x10^7	6,87x10^6	41	1A/1B
22	11-6-2014	adenoom	Canine	1310639	12-11-2013	Bolke/Grunsven	Collagenase	430.000	20.200	5	1A/1B
23	11-6-2014	adenoom	Canine	1100247	15-2-2011	Bernard/Kiera	Collagenase	1,05x10^7	4,64x10^6	44	1A/1B
24	11-6-2014	adenoom	Canine	1302453	12-3-2013	Preisig/Qailash	Collagenase	192.000	154.000	8	1A/1B
25	11-6-2014	adenoom	Canine	909930	26-1-2010	Loomans/Bigfoot	Collagenase	7,45x10^6	743.000	10	1A/1B
26	11-6-2014	adenoom	Canine	1306776	25-3-2014	Terpstra/Charly	Collagenase	460.000	152.00	33	1A/1B
27	11-6-2014	adenoom	Canine	910051	2-2-2010	Nijholt/Hunya	Collagenase	359.000	75800	21	1A/1B
28	18-6-2014	hypofyse	Canine	892190	22-4-2014	GDL	Trypsine	600.000	131.500	22	1A/1B
29	18-6-2014	hypofyse	Canine	903728	22-4-2014	GDL	Trypsine	1,08x10^6	258.000	24	1A/1B
30	18-6-2014	hypofyse	Canine	898473	22-4-2014	GDL	Trypsine	346.500	113.500	33	1A/1B
31	18-6-2014	hypofyse	Canine	911364	22-4-2014	GDL	Trypsine	1,11x10^6	273.000	25	1A/1B
32	4-7-2014	hypofyse	Canine	918911	23-4-2014	GDL	Trypsine	1,69x10^6	245.000	12	1A/1B
33	4-7-2014	hypofyse	Canine	989503	16-4-2014	GDL	Trypsine	3,36x10^6	1,02x10^6	30	1A/1B
34	9-7-2014	hypofyse	Canine	920738	9-4-2014	GDL	Trypsine	871.200	245.000	31	1A/1B
35	9-7-2014	hypofyse	Canine	922994	14-4-2014	GDL	Trypsine	1,16x10^6	290.500	25	1A/1B
36	9-7-2014	hypofyse	Canine	1399617	7-4-2014	GDL	Trypsine	5,75x10^6	2,46x10^6	43	1A/1B
37	25-7-2014	hypofyse	Canine		26-3-2013	GDL	Trypsine	2,95x10^6	900.000	31	2A/2B
37	25-7-2014	hypofyse	Canine		26-3-2013	GDL	Trypsine	2,95x10^6	900.000	31	2A/2B
38	6-8-2014	hypofyse	Canine	854476	16-7-2014	GDL	Trypsine	6,1x10^6	1,06x10^6	17	2A/2B
39	28-8-2014	hypofyse	Canine	1003474	24-7-2010	labrador pup	Trypsine	3,83x10^6	440.000	11	2A/2B
40	8-9-2014	hypofyse	Canine	944904	19-8-2014	GDL	Trypsine	1,24x10^6	201.000	19	2A/2B
41	11-9-2014	Adenoom	Feline	1407203	10-9-2014	Suberbielle	Collagenase	1,13x10^7	580.000	5	2A/2B
42	11-9-2014	Hypofyse	Canine	903752	10-9-2014	GDL	Trypsine	2,65x10^6	341.000	14	2A/2B
43	16-9-2014	hypofyse	Canine	921653	15-9-2014	GDL	Trypsine	1850000	351500	19	2A/2B
44	18-9-2014	Adenoom	Feline	1408679	17-9-2014	Cosmo / Engel	Trypsine	3395000	149000	4	2A/2B
45	25-9-2014	adenoom	Feline	1408510	24-9-2014	Foglia / Trifone	Collagenase	392000	76000	2	2A/2B
46	25-9-2014	hypofyse	Canine	897086	24-9-2014	GDL	Trypsine	2,46x10^6	467500	19	2A/2B

Table D1: Characteristics and cell counts of the samples used for FACS analysis, as described in chapter 5. Medium 1A consisted of 1 liter DMEM 1:1/F12 containing 5 mg transferrin, 5 mg Insulin, 1% penicillin streptomycin, 1 mg catalase and 1 g NaHCO3. Medium 1B was HBSS, containing 2% FCS, 2µg/ml propidium iodide (PI), 0.4% DNase and 0.025% EDTA. Medium 2A consisted of DMEM Glutamax, 2% FCS+ 10 mM Hepes + 0.4% Insulin and medium 2B was HBSS enriched with 2% FCS and 2µg/ml PI.

Sample nr.	Hoechst incubation (min)	FACS	SP proportion (%)	SP proportion (control) (%)	Collection method	Short-term storage	Storage at -70°C in	Date RNA isolation
1	60							
2	60							
3	60							
4	60							
5								
6	60	FACSAria II	0,5	0,2	RNA later	Kept on ice	RNA later	10-6-2014
7	60	FACSAria II	0,4	0,1	RNA later	Kept on ice	RNA later	10-6-2014
8	90	FACSAria II	1,8	0,3	RNA later	Kept on ice	75 µl RLT buffer +ßMC	16-6-2014
9	90	FACSAria II	0,2	0	RNA later	Kept on ice	75 µl RLT buffer +ßMC	16-7-2014
10	90	FACSAria II	0,2	0	RNA later	Kept on ice	75 µl RLT buffer +ßMC	16-7-2014
11	90	FACSAria II	0,1	0	RNA later	Kept on ice	75 µl RLT buffer +ßMC	16-7-2014
12	90	FACSAria II	1,2	0,8	RNA later	Kept on ice	75 µl RLT buffer +ßMC	10-6-2014
13	90	FACSAria II	0,3	0	RNA later	Kept on ice	75 µl RLT buffer +ßMC	16-7-2014
14	90	FACSAria II	0,7	0,1	RNA later	Kept on ice	75 µl RLT buffer +ßMC	16-7-2014
15	90	FACSAria II	0,5	0	RNA later	Kept on ice	75 µl RLT buffer +ßMC	16-7-2014
16	90	FACSAria II	0,1	0,1	RNA later	Kept on ice	75 µl RLT buffer +ßMC	
17	90	FACSAria II	3,6	0,1	RNA later	Kept on ice	75 µl RLT buffer +ßMC	
18	90	FACSAria II	0,5	0,1	RNA later	Kept on ice	75 µl RLT buffer +ßMC	
19	90	FACSAria II	4,1	1	RNA later	Kept on ice	75 µl RLT buffer +ßMC	
20	90	FACSAria II						
21	90	FACSAria II	0	0	RNA later	Kept on ice	75 µl RLT buffer +ßMC	7-7-2014
22	90	FACSAria II						
23	90	FACSAria II	1,6	0,4	RNA later	Kept on ice	350 µl RLT buffer +ßMC	7-7-2014
24	90	FACSAria II	2,4	0	RNA later	Kept on ice	75 µl RLT buffer +ßMC	
25	90	FACSAria II	1,2	1,3	RNA later	Kept on ice	75 µl RLT buffer +ßMC	
26	90	FACSAria II	0,3	0	RNA later	Kept on ice	75 µl RLT buffer +ßMC	
27	90	FACSAria II	4	0	RNA later	Kept on ice	75 µl RLT buffer +ßMC	
28	90	FACSAria II						
29	90	FACSAria II						
30	90	FACSAria II						
31	90	FACSAria II						
32	90	FACSAria II	0,1	0	RLT buffer +ßMC	Snap frozen	350 µl RLT buffer +ßMC	7-7-2014
33	90	FACSAria II	0,2	0,1	RLT buffer +ßMC	Snap frozen	350 µl RLT buffer +ßMC	7-7-2014
34	90	FACSAria II	2	0,1	RLT buffer +ßMC	Snap frozen	350 µl RLT buffer +ßMC	11-7-2014
35	90	FACSAria II	1,1	0	RLT buffer +ßMC	Snap frozen	350 µl RLT buffer +ßMC	16-7-2014
36	90	FACSAria II	0	0	RLT buffer +ßMC	Snap frozen	350 µl RLT buffer +ßMC	16-7-2014
37	90	FACSAria II			RLT buffer	Kept on ice	RNA isolation	25-7-2014
37	90	FACSAria II			Empty tube	Kept on ice	RNA isolation	25-7-2014
38	90	FACSAria II			Empty tube	Kept on ice	RNA isolation	6-8-2014
39	90	BD Influx			RLT buffer +ßMC	Kept on ice	RNA isolation	28-8-2014
40	60	BD Influx			RLT buffer +ßMC	Kept on ice	RNA isolation	8-9-2014
41	90	BD Influx			RLT buffer +ßMC	Kept on ice	RNA isolation	10-9-2014
42	90	BD Influx			RLT buffer +ßMC	Kept on ice	RNA isolation	10-9-2014
43	90	BD Influx			RLT buffer +ßMC	Kept on ice	350 µl RLT buffer +ßMC	22-9-2014
44	90	BD Influx			RLT buffer +ßMC	Kept on ice	350 µl RLT buffer +ßMC	22-9-2014
45	90	BD Influx			RLT buffer +ßMC	Kept on ice	350 µl RLT buffer +ßMC	26-9-2014
46	90	BD Influx			RLT buffer +ßMC	Kept on ice	350 µl RLT buffer +ßMC	26-9-2014

Table D1 (Continuation): SP proportion is given in percentage SP cells of the life cells. β –ME, β-Mercaptoethanol;

Sample	Side population	Nanadrop		Bioanalyzer		Main population	Nanodrop		Bioanalyzer	
nr.	Sorted cells	Quantity (ng/µl)	Quality	Quantity (pg/µ)	Quality (RIN)	Sorted cells	Quantity (ng/µ)	Quality	Quantity (pg/µ)	Quality (RIN)
1										
2										
3										
4										
5										
6	6.300	9,5	1,38			9.000	4,4	1,86		
7	1.915	6,8	2,07			1.915	6,8	2,07		
8	7.219	7,4	1,48	88	2,9	4.472	12,6	1,6	16	1
9	4.472	12,6	1,6	16	1	503	4,4	1,2	34	1
10	1.800	5,9	1,46			618	3	2		
11	720	4,2	1,38	72	5,7	1.200	5,2	1,75	18	1
12	13.000	8,2	1,67	27	1	66.000	9,6	1,48	75	1,2
13	28.000	4,7	2,07	100	6,2	14.000	3,9	1,39	0	N/A
14	9.800	4,1	1,8	952	N/A	5.000	4	1,36	75	5,9
15	80.000	4,1	1,09			51.000	5,5	1,69		
16	35					358				
17	45.000					5.000				
18	12.000					7.000				
19	48.000					10.000				
20										
21	50.000	3,5	2,13	2708	1	100.000	6,8	1,28	120	1,1
22										
23	140.000	8,8	1,77	2158	8,1	150.000	8,5	1,51	7230	N/A
24	4.034					2.398				
25	608.000					28.000				
26	2.800					4.600				
27	7.800					12.000				
28	1.611					1.583				
29	9.461					5.720				
30	14.000					3.600				
31	4.124					1.224				
32	1800	4,5	1,53	1314	N/A	7000	5,5	1,34	134	1
33	10.000	10,1	1,36	65	1	8700	4,1	1,52	553	1,1
34	7100	4,8	1,68			4000	5,5	2,06		
35	2900	7,7	1,69	0	N/A	2700	5,9	1,21	0	N/A
36	64	6	3,67	0	N/A	2938	5,5	1,45	0	N/A
37	5981	x	x	46	1	30.000	x	x	102	1,3
37	3677	x	x	49	1	25.000	x	x	63	6,5
38	245	5,2	1,21			5600	5,7	1,31		
39	1597	cytospin	cytospir	cytospin	cytospin	1567	6,1	1,58	32	1
40	1953	6,5	1,08	17	1	12858	8,7	1,65	152	6,7
41	768	3,9	1,72	5	N/A	12888	20,4	1,5	409	2,2
42	17123	2,6	2,84	63	N/A	16329	4,7	4,63	71	4,6
43	1449	18,48	1,39			14439	22,85	1,39		
44	219	3,28	1,20			4380	22,92	1,47		
45	154	2,57	2,08			216	2,42	2,27		
46	43983	39,4	1,48			46065	9,76	1,81		

Table D1 (Continuation): RNA isolation was performed on the SP and MP samples. RNA quality and quantity was measured on the Nandrop and Agilent Bioanalyzer. abbreviations: N/A, not available;

											HMBS cat			RPL17 cat			GAPDH cat	
Sample	Donor	Patient	FACS	Tissue	Species	FACSAria II	Fresh/	MP/SP	Input	RIN	463,70%			-55,00%			-61,40%	
nr.		nr.	date			Instrument	frozen			value	sample 1	sample 2	Mean	sample 1	sample 2	Mean	sample 1	sample 2
	Standaard	S1		1							33,27	36,83	35,05	33,44	33,79	33,62	35,17	34,24
	Standaard	S2		1:4							34,82	37,34	36,08	N/A	39,39	N/A	34,93	N/A
	Standaard	S3		1:16							N/A	N/A	N/A	34,32	33,20	33,76	34,35	35,57
	Standaard	S4		1:64							35,81	N/A	N/A	32,13	31,47	31,80	34,00	33,25
	Standaard	S5		1:256							39,34	N/A	N/A	30,37	30,33	30,35	31,06	31,44
	Standaard	S6		1:1024							N/A	N/A	N/A	28,26	28,22	28,24	29,62	29,61
	Standaard	S7		1:4096							N/A	N/A	N/A	25,56	25,60	25,58	27,29	27,46
	Standaard	S8		0							N/A	N/A	N/A	23,63	23,58	23,61	25,10	25,50
37	GDL	?	25-7-2014	hypofyse	Canine	FACSAria II	Frozen	SP	552	1	N/A	N/A	N/A	31,85	30,93	31,39	35,13	
37			25-7-2014	hypofyse	Canine	FACSAria II	Frozen	MP	1224	1,3	39,29	35,14	37,22	30,72	30,56	30,64	32,03	
37	GDL	?	25-7-2014	hypofyse	Canine	FACSAria II	Frozen	SP	588	1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	
37			25-7-2014	hypofyse	Canine	FACSAria II	Frozen	MP	756	6,5	N/A	N/A	N/A	32,80	33,21	33,01	34,13	
39	labrador pup	1003474	28-8-2014	hypofyse	Canine	FACSAria II	Frozen	MP	384	1	N/A	N/A	N/A	29,35	29,49	29,42	32,07	
40	GDL	944904	8-9-2014	hypofyse	Canine	FACSAria II	Frozen	SP	204	1	36,15	N/A	N/A	30,78	30,61	30,70	33,41	
40			8-9-2014	hypofyse	Canine	FACSAria II	Frozen	MP	1824	6,7	35,17	35,61	35,39	26,20	25,95	26,08	28,31	
41	Waryo / Suberbielle	1407203	10-9-2014	adenoom	Feline	BD Influx	Fresh	SP	60	N/A	N/A	N/A	N/A	33,09	31,04	32,07	32,81	
41			10-9-2014	adenoom	Feline	BD Influx	Fresh	MP	4908	2,2	33,46	N/A	N/A	28,12	27,98	28,05	30,27	
42	GDL	903752	10-9-2014	hypofyse	Canine	BD Influx	Fresh	SP	756	N/A	N/A	36,33	N/A	28,28	28,73	28,51	30,38	
42			10-9-2014	hypofyse	Canine	BD Influx	Fresh	MP	852	4,6	N/A	37,40	N/A	26,55	26,74	26,65	28,42	
43	GDL (104879-1)	921653	16-9-2014	hypofyse	Canine	BD Influx	Fresh	SP			N/A	N/A	N/A	30,25	31,12	30,69	32,37	
43			16-9-2014	hypofyse	Canine	BD Influx	Fresh	MP			37,25	38,19	37,72	25,87	25,72	25,80	27,54	
44	Cosmo / Engel	1408679	18-9-2014	adenoom	Feline	BD Influx	Fresh	SP			N/A	N/A	N/A	34,10	34,83	34,47	35,97	
44			18-9-2014	adenoom	Feline	BD Influx	Fresh	MP			N/A	33,59	N/A	28,06	28,59	28,33	32,34	
45	Foglia / Trifone	1408510	25-9-2014	adenoom	Feline	BD Influx	Fresh	SP			N/A	N/A	N/A	N/A	N/A	N/A	N/A	
45			25-9-2014	adenoom	Feline	BD Influx	Fresh	MP			N/A	N/A	N/A	32,97	32,76	32,87	34,43	
46	GDL (104879-1)	897086	25-9-2014	hypofyse	Canine	BD Influx	Fresh	SP			34,13	34,07	34,10	24,29	24,03	24,16	25,70	
46			25-9-2014	hypofyse	Canine	BD Influx	Fresh	MP			34,50	34,26	34,38	23,76	23,21	23,49	24,86	

Table D2: Sample characteristics and Ct values of the qPCR study as described in chapter 6. Quantitative PCR was done on a subset of the sorted samples, sorted by two different FACS instruments. Canine (hydroxymethylbilane synthase (hmbs), tata box binding protein (tbp), and ,tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (ywhaz)) and feline (hmbs, ribosomal protein L 17 (rpl17), Glyceraldehyde 3-phosphate dehydrogenase (gapdh)) reference genes were tested, as well as canine dopamine receptor D2 (Drd2), sex determining region Y-box 2 (Sox2) and paired box protein 7 (Pax7). Of the samples 43, 44, 45 and 46, RNA quantity and quality was not measured. All measurements were done in duplo, except for gapdh.

	HMBS dog)			Tbp (dog)			YWHAZ (dog)			Mean (Dog)	Drd2 (dog)				Sox2 (dog)				Pax7 (dog)			
	209,80%			657,90%			-68,30%				110,60%	2,106			193,70%				-23,10%			
nr.	sample 1	sample 2	Mean	sample 1	sample 2	Mean	sample 1	sample 2	Mean		sample 1	sample 2	Mean	Drd2-ref	sample 1	sample 2	Mean	Sox2-ref	sample 1	sample 2	Mean	Pax7-ref
	30,33	31,04	30,69	30,69	N/A	N/A	25,94	28,55	27,25	28,97	27,67	27,38	27,53	-1,44	31,96	31,12	31,54	2,58	N/A	34,38	N/A	N/A
	32,74	33,23	32,99	33,91	32,82	33,37	27,78	29,92	28,85	31,73	28,75	29,68	29,22	-2,52	32,89	33,95	33,42	1,69	34,58	34,22	34,40	2,67
	32,40	33,83	33,12	35,12	35,20	35,16	30,54	32,03	31,29	33,19	31,42	32,06	31,74	-1,45	34,51	35,2	34,86	1,67	N/A	N/A	N/A	N/A
	N/A	34,74	N/A	35,74	N/A	N/A	31,59	35,50	33,55	33,55	32,15	34,54	33,35	-0,20	34,87	35,84	35,36	1,81	N/A	N/A	N/A	N/A
	N/A	N/A	N/A	N/A	37,69	37,69	N/A	N/A	N/A	37,69	N/A	34,49	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	3,79	N/A	N/A	N/A
	N/A	N/A	N/A	36,21	N/A	N/A	36,95	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	N/A	N/A	N/A	N/A	36,35	N/A	6,72	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	4,14	N/A	N/A	N/A
37	N/A	N/A	N/A	35,81	N/A	N/A	32,48	32,93	32,71	32,71	N/A	N/A	N/A	N/A	N/A	36,62	N/A	N/A	3,24	N/A	N/A	N/A
37	33,87	N/A	N/A	N/A	N/A	N/A	31,93	32,52	32,23	32,23	N/A	36,94	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
37	N/A	N/A	N/A	N/A	N/A	N/A	N/A	34,21	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
37	N/A	N/A	N/A	N/A	N/A	N/A	N/A	37,06	N/A	N/A	N/A	N/A	N/A	N/A	38,13	N/A	N/A	N/A	3,77	N/A	N/A	N/A
39	33,38	33,40	33,39	N/A	33,55	N/A	33,01	31,65	32,33	32,86	N/A	N/A	N/A	N/A	35,93	33,47	34,70	1,84	33,08	N/A	N/A	N/A
40	N/A	N/A	N/A	N/A	N/A	N/A	31,50	31,34	31,42	31,42	32,93	34,06	33,50	2,08	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
40	31,49	31,48	31,49	32,26	32,56	32,41	27,49	27,48	27,49	30,46	30	29,98	29,99	-0,47	30,29	30,45	30,37	-0,09	35,1	33,97	34,54	4,08
41	N/A	N/A	N/A	33,02	35,17	34,10	36,96	N/A	N/A	34,10	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
41	N/A	33,21	N/A	N/A	N/A	N/A	29,56	29,42	29,49	29,49	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	4,75	N/A	N/A	N/A
42	33,45	36,72	35,09	32,56	32,67	32,62	29,48	29,02	29,25	32,32	31,02	31,38	31,20	-1,12	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
42	33,30	32,05	32,68	33,02	31,38	32,20	28,54	28,46	28,50	31,13	31,32	30,92	31,12	0,00	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
43	34,47	34,96	34,72	N/A	33,80	N/A	32,20	33,56	32,88	33,80	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	5,27	4,38	4,83	-28,97
43	32,71	31,45	32,08	31,34	32,09	31,72	27,12	26,74	26,93	30,24	31,33	30,59	30,96	0,72	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
44	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
44	N/A	N/A	N/A	N/A	N/A	N/A	30,50	30,44	30,47	30,47	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
45	N/A	N/A	N/A	26,12	N/A	N/A	18,47	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
45	N/A	N/A	N/A	N/A	N/A	N/A	N/A	38,22	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
46	28,77	29,63	29,20	30,03	29,84	29,94	25,41	24,76	25,09	28,07	27,17	27,26	27,22	-0,86	33,11	32,04	32,58	4,50	N/A	N/A	N/A	N/A
46	28,66	29,14	28,90	30,01	29,73	29,87	24,77	24,46	24,62	27,80	26,48	27,05	26,77	-1,03	31,25	31,82	31,54	3,74	N/A	34,49	N/A	N/A

Table D2 (continuation)

				Hmbs			tbp			ywahz			Mean
				101,90%			91,10%			98,20%			94,65%
				2,019			1,911			1,982			1,9465
				sample 1	sample 2	Mean	sample 1	sample 2	Mean	sample 1	sample 2	Mean	
Donor	patient num	tissue	name										
kruising	847186	hypofyse	1	21,21	21,55	21,38	24,03	23,83	23,93	18,89	18,87	18,88	21,40
Mongrel	46715	hypofyse	2	22,08	22,04	22,06	24,22	24,53	24,37	19,29	19,16	19,22	21,89
Beagle	1206435	hypofyse	3	22,09	22,28	22,18	25,29	25,31	25,30	20,13	20,53	20,33	22,60
Beagle		hypofyse	4	21,49	21,34	21,41	24,37	23,97	24,17	18,93	18,84	18,88	21,49
GDL		hypofyse	5	21,12	20,66	20,89	24,28	24,34	24,31	18,84	18,75	18,79	21,33
Beagle	1206430	hypofyse	6	21,39	21,55	21,47	24,84	25,08	24,96	19,55	19,56	19,55	21,99
GDL		hypofyse	7	21,72	21,4	21,56	25,65	25,84	25,74	21,11	20,16	20,63	22,64
Beagle	1206429	hypofyse	8	22,03	21,93	21,98	25,07	25,28	25,17	19,78	20,28	20,03	22,39
Mongrel	843598	hypofyse	9	21,86	21,69	21,77	24,51	24,59	24,55	19,2	19,27	19,23	21,85
Kruising	846520	hypofyse	10	21,75	22,03	21,89	24,89	24.75	24,89	20,19	20,36	20,27	22,35
nr 3		hypofyse	11	21,23	21,33	21,28	25,36	25,33	25,34	20,23	20,42	20,32	22,32
Beagle		hypofyse	12	21,57	21,55	21,56	25,39	25,28	25,33	19,69	19,85	19,77	22,22
King grey		hypofyse	13	21,58	21,36	21,47	24,27	24,17	24,22	18,44	18,2	18,32	21,34
Beagle	1206427	hypofyse	14	21,27	21,46	21,36	24,64	25,14	24,89	19,37	19,23	19,30	21,85
Linda		hypofyse	15	21,85	21,61	21,73	24,51	24,5	24,50	19,84	19,97	19,90	22,05
Beagle	1206433	hypofyse	16	22,28	21,53	21,90	24,72	24,54	24,63	19,4	19,3	19,35	21,96
Beagle	1206436	hypofyse	17	21,78	21,45	21,61	25,14	24,81	24,97	20,03	19,85	19,94	22,18
Beagle	1206426	hypofyse	18	22,11	22,33	22,22	24,64	25,27	24,95	19,59	19,86	19,72	22,30
Beagle	1206432	hypofyse	19	21,68	21,45	21,56	25,18	25,11	25,14	19,67	20,03	19,85	22,19
Beagle	1206431	hypofyse	20	21,64	21,56	21,60	25,12	25,23	25,17	19,97	19,84	19,90	22,23
Kwakkel	227484	Adenoom	1	22,09	21,38	21,73	25,4	25,5	25,45	20,6	20,77	20,68	22,62
ten Wolde	402572	Adenoom	2	20,76	20,65	20,70	23,31	23,79	23,55	18,51	18,37	18,44	20,90
Bakker	402190	Adenoom	3	23,39	23,43	23,41	26,42	26,68	26,55	21,56	21,6	21,58	23,85
Bonnier	405068	Adenoom	4	22,14	21,73	21,93	24,85	25,05	24,95	20,07	19,87	19,97	22,28
vanDieren	509455	Adenoom	5	21,95	21,71	21,83	24,47	25,03	24,75	18,47	18,87	18,67	21,75
Kerkdijk	509253	Adenoom	6	21,61	21,22	21,41	24,33	24,67	24,50	19,36	19,2	19,28	21,73
Hagoort	509659	Adenoom	7	21,84	21,35	21,59	23,92	24,38	24,15	19,11	19,04	19,07	21,61
Alberami	510921	Adenoom	8	22,78	22,99	22,88	25,01	24,95	24,98	20,44	20,32	20,38	22,75
Zadel	510953	Adenoom	9	20,21	20,22	20,21	N/A	N/A	N/A	19,58	18,79	19,18	19,70
Bantjes	601011	Adenoom	10	21,89	21,64	21,76	N/A	N/A	N/A	19,96	20,11	20,03	20,90
Ceroni	603058	Adenoom	11	21,39	21,16	21,27	N/A	N/A	N/A	19,21	19,64	19,42	20,35
Boot	602511	Adenoom	12	20,7	20,42	20,56	N/A	N/A	N/A	19,11	19,28	19,19	19,88
Knobloch	606441	Adenoom	13	22,23	22,23	22,23	N/A	N/A	N/A	19,02	19,2	19,11	20,67
Veen	606211	Adenoom	14	22,2	21,67	21,93	N/A	N/A	N/A	19,6	19,64	19,62	20,78
Marx	604429	Adenoom	15	22,82	22,76	22,79	N/A	N/A	N/A	20,06	19,77	19,91	21,35
Paeps	609333	Adenoom	16	22,04	21,71	21,87	N/A	N/A	N/A	19	19,47	19,23	20,55
van der leel	609507	Adenoom	17	20,6	20,39	20,49	25,81	25,77	25,79	19,25	19,13	19,19	21,82
Otte	606781	Adenoom	18	20,58	20,44	20,51	23,35	23,59	23,47	18,27	18,02	18,14	20,71
de Gilde	701225	Adenoom	19	20,59	20,48	20,53	23,75	23,92	23,83	19,4	19,26	19,33	21,23
Raeijmaeke	702432	Adenoom	20	22,38	22,43	22,40	26,32	26,25	26,28	20,98	21,15	21,06	23,25

Table D3: qPCR data of the PC2 study described in chapter 7. RT-qPCR was done for the genes Dopamine receptor D2 (Drd2), hydroxymethylbilane synthase (hmbs), Paired box 7 (Pax7), Prohormone converstase 2 (PC2), Proopiomelanocortin (POMC), SRY (sex determining region Y)-box 2 (Sox2), tata box binding protein (tbp), Thrombospondin 2 (Thbs2) and ,tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (ywhaz).

	Sox2				Drd2				Pcsk2			
	92,50%	1,925			110%	2,1			102,20%	2,022		
	sample 1	sample 2	Mean	Dct	sample 1	sample 2	Mean	Dct	sample 1	sample 2	Mean	Dct
name												
1	20,01	20,18	20,09	-1,30	21,33	21,22	21,27	-0,12	19,03	18,99	19,01	-2,39
2	21,09	21,21	21,15	-0,74	21,3	21,43	21,36	-0,52	20,41	20,28	20,34	-1,54
3	21,43	21,31	21,37	-1,23	22,86	22,96	22,91	0,31	21,04	21,01	21,02	-1,58
4	19,8	19,78	19,79	-1,70	20,48	20,51	20,49	-0,99	20,35	20,09	20,22	-1,27
5	20,45	20,68	20,56	-0,77	21,01	21,14	21,07	-0,26	20,99	21,05	21,02	-0,31
6	20,11	20,12	20,11	-1,88	21,09	21,14	21,11	-0,88	19,46	19,62	19,54	-2,46
7	21	21,07	21,03	-1,61	22,31	22,52	22,41	-0,23	22,56	22,57	22,56	-0,08
8	21,08	21,17	21,12	-1,27	21,51	21,53	21,52	-0,87	20,42	20,24	20,33	-2,06
9	21,09	20,81	20,95	-0,90	21,32	21,31	21,31	-0,54	20,56	20,43	20,49	-1,36
10	21,08	20,53	20,80	-1,55	21,74	21,77	21,75	-0,60	19,53	20	19,76	-2,59
11	20,54	20,53	20,53	-1,78	21,04	21,26	21,15	-1,17	20,9	20,58	20,74	-1,58
12	20,5	20,46	20,48	-1,74	21,73	21,96	21,84	-0,38	21,18	20,97	21,07	-1,15
13	21,18	21,1	21,14	-0,20	24,63	24,96	24,79	3,46	24,02	23,49	23,75	2,42
14	20,3	20,31	20,30	-1,55	21,43	21,43	21,43	-0,42	20,05	19,79	19,92	-1,93
15	21	20,86	20,93	-1,12	21,45	21,46	21,45	-0,59	20,38	20,85	20,61	-1,43
16	20,39	20,66	20,52	-1,44	21,42	21,59	21,50	-0,46	20,82	20,73	20,77	-1,19
17	21,19	20,97	21,08	-1,10	21,96	21,67	21,81	-0,36	20,78	20,49	20,63	-1,54
18	21,27	21,52	21,39	-0,90	22,03	22,17	22,10	-0,20	21,06	21,32	21,19	-1,11
19	20,54	20,62	20,58	-1,61	21,27	21,23	21,25	-0,94	20,37	20,21	20,29	-1,90
20	21,02	21,25	21,13	-1,09	21	20,84	20,92	-1,31	21,09	21,01	21,05	-1,18
1	21,21	21,16	21,18	-1,44	22,87	22,75	22,81	0,19	21,44	21,32	21,38	-1,24
2	20,41	20,61	20,51	-0,39	25,79	25,39	25,59	4,69	21,53	21,61	21,57	0,67
3	22,35	22,77	22,56	-1,29	24,64	24,5	24,57	0,72	21,87	21,92	21,89	-1,95
4	22,6	22,72	22,66	0,38	20,92	21,04	20,98	-1,30	19,65	19,3	19,47	-2,81
5	21,96	22,06	22,01	0,26	24,4	24,64	24,52	2,77	22,72	22,46	22,59	0,84
6	22,23	22,36	22,29	0,56	23,67	23,55	23,61	1,88	17,74	17,44	17,59	-4,14
7	22,16	22,1	22,13	0,52	26,43	26,5	26,46	4,86	21,89	21,71	21,80	0,19
8	23,7	23,53	23,61	0,87	27,41	27,15	27,28	4,53	24,14	23,45	23,79	1,04
9	21,04	20,81	20,92	1,23	23,63	23,31	23,47	3,77	20,79	20,56	20,67	0,98
10	21,97	21,6	21,78	0,88	25,48	25,3	25,39	4,49	20,65	20,69	20,67	-0,23
11	22,27	22,28	22,27	1,93	24,36	24	24,18	3,83	21,35	21,44	21,39	1,05
12	18,94	18,87	18,90	-0,97	22,03	22,03	22,03	2,15	19,84	19,91	19,87	0,00
13	21,74	21,73	21,73	1,07	30,12	29,64	29,88	9,21	26,32	26,52	26,42	5,75
14	21,01	20,8	20,90	0,13	20,68	20,27	20,47	-0,30	22,34	22,4	22,37	1,59
15	21,39	20,97	21,18	-0,17	20,57	20,03	20,30	-1,05	19,08	18,92	19,00	-2,35
16	20,64	20,77	20,70	0,15	26,74	26,46	26,60	6,05	21,41	21,1	21,25	0,70
17	22,5	22,37	22,43	0,61	25,97	26	25,98	4,16	25,15	25,01	25,08	3,26
18	19,74	19,7	19,72	-0,99	27,68	27,24	27,46	6,75	26,47	26,96	26,71	6,01
19	20,55	20,71	20,63	-0,60	24,39	24,09	24,24	3,01	24,3	24,12	24,21	2,98
20	24,17	24,35	24,26	1,01	28,44	28,57	28,50	5,25	25,03	25,04	25,03	1,78

Table D3 (continuation)

	Thbs2				POMC				pax7			
	93,90%	1,939			107,10%	2,071			93,50%	1,935		
name	sample 1	sample 2	Mean	Dct	sample 1	sample 2	Mean	Dct	sample 1	sample 2	Mean	Dct
1	26,31	26,14	26,22	4,83	16,61	15,36	15,97	-5,42	24,16	24,72	24,44	3,04
2	26,52	26,2	26,36	4,47	18,38	18,21	18,29	-3,59	27,02	27,18	27,10	5,21
3	27,51	27,4	27,45	4,85	18,72	18,1	18,41	-4,20	27,99	27,67	27,83	5,22
4	26,41	26,53	26,47	4,98	17,88	18,02	17,95	-3,54	25,51	25,16	25,33	3,84
5	27,05	27,02	27,03	5,70	18,5	18,43	18,46	-2,87	28,06	27,43	27,74	6,41
6	26,76	27,06	26,91	4,91	17,3	17,34	17,32	-4,67	24,33	24,77	24,55	2,55
7	24,17	24,14	24,15	1,51	18,29	18,06	18,17	-4,47	28,12	28,03	28,07	5,43
8	27,46	28,02	27,74	5,34	17,19	17,02	17,10	-5,29	24,78	25,87	25,32	2,92
9	27,56	27,78	27,67	5,82	17,46	17,76	17,61	-4,24	26,92	27,06	26,99	5,14
10	26,11	25,68	25,89	3,54	17,96	18,54	18,25	-4,10	26,33	26,37	26,35	4,00
11	28,56	28,25	28,40	6,09	19,14	19,02	19,08	-3,24	29,72	30,61	30,16	7,85
12	N/A	28,31	N/A	N/A	19,42	19,35	19,38	-2,84	29,16	28,99	29,07	6,85
13	27,74	27,65	27,69	6,36	13,35	13,59	13,47	-7,87	32,01	31,76	31,88	10,55
14	27,24	27,32	27,28	5,43	18,06	17,05	17,55	-4,30	25,81	26,18	25,99	4,14
15	29,27	28	28,63	6,58	18,49	18,44	18,46	-3,58	9,69	N/A	N/A	N/A
16	27,23	27,04	27,13	5,17	16,99	16,74	16,86	-5,10	N/A	N/A	N/A	N/A
17	27,79	27,26	27,52	5,35	18,41	18,99	18,70	-3,48	27,97	28,01	27,99	5,81
18	27,87	27,88	27,87	5,58	19,59	19,77	19,68	-2,62	28,43	28,71	28,57	6,27
19	27,28	27,07	27,17	4,99	18,04	17,32	17,68	-4,51	26,13	26,29	26,21	4,02
20	26,33	26,13	26,23	4,00	19,49	20,06	19,77	-2,45	27,73	27,87	27,80	5,57
1	28,62	28,12	28,37	5,75	17,64	18,2	17,92	-4,70	27,92	28,71	28,31	5,69
2	22,95	23,21	23,08	2,18	17,76	17,13	17,44	-3,46	30,1	30,03	30,06	9,17
3	30,88	31,25	31,06	7,22	18,79	19,08	18,93	-4,91	28,49	29,18	28,83	4,99
4	31,63	31,02	31,32	9,04	16,23	15,27	15,74	-6,54	27,6	27,57	27,58	5,30
5	25,8	26,04	25,92	4,17	19,71	20,19	19,95	-1,80	N/A	34,3	N/A	N/A
6	26,3	26,43	26,36	4,63	18,44	18,99	18,71	-3,02	30,73	31,2	30,96	9,23
7	29,86	29,75	29,80	8,20	16,14	15,63	15,88	-5,72	38,67	35,37	36,98	15,38
8	26,29	26,51	26,40	3,65	21,48	21,2	21,34	-1,41	30,98	32,29	31,63	8,88
9	25,55	25,41	25,48	5,78	20,58	21,04	20,81	1,11	28,93	29,29	29,11	9,41
10	28,46	28,4	28,43	7,53	18,82	19,78	19,29	-1,61	32,22	31,13	31,67	10,77
11	25,58	25,93	25,75	5,41	20,35	20,26	20,30	-0,04	28,1	28,46	28,28	7,93
12	27,67	28,02	27,84	7,97	19,24	18,59	18,91	-0,96	25,3	24,94	25,12	5,24
13	27,51	27,32	27,41	6,74	15,18	15,45	15,31	-5,36	34,01	33,36	33,68	13,01
14	30,62	30,41	30,51	9,74	19,38	20,39	19,88	-0,90	N/A	31,87	N/A	N/A
15	29,19	29,27	29,23	7,88	13,85	14,24	14,04	-7,31	30,74	30	30,37	9,02
16	30,17	30,02	30,09	9,54	16,8	17,36	17,08	-3,48	30,05	29,65	29,85	9,30
17	30,1	29,83	29,96	8,14	15,2	15,48	15,34	-6,49	32,35	34,05	33,19	11,36
18	30,01	30,11	30,06	9,35	20,73	20,79	20,76	0,05	27,78	27,73	27,75	7,05
19	26,23	26,02	26,12	4,89	17,44	17,28	17,36	-3,87	30,48	30,35	30,41	9,18
20	24,18	24,22	24,20	0,95	21,76	21,74	21,75	-1,50	34,54	36,13	35,33	12,07

Table D3 (continuation)

Livak Method

	hmbs		tbp		ywahz		Mean Ref		Sox2		Drd2		Pcsk2		Thbs2		POMC	
	101.90%	2,019	91,10%	1,911	98,20%	1,982			92,50%	1,925	110%	2,11	102,20%	2,022	93,90%	1,939	107,10%	2,071
	Hypofyse	Adenoom	Hypofyse	adenoom	hypofyse	Adenoom	hypofyse	adenoom	Hypofyse	Adenoom	Hypofyse	Adenoom	Hypofyse	Adenoom	Hypofyse	Adenoom	Hypofyse	Adenoom
Mean Ct	21,64293	21,58685	24,82434	24,8354	19,60232	19,55844	22,025	21,40766	20,75035	21,58577	21,65799	24,58353	20,69447	22,0128	27,0264	27,77346	17,85131	18,20295
ΔCt									-1,27465	0,178101	-0,36701	3,175868	-1,33053	0,605135	5,00145	6,365795	-4,17369	-3,20472
ΔΔCT									0	1,452753	0	3,542878	0	1,935662	0	1,364349	0	0,968968
E^-ΔΔCt									1	0,386182	1	0,070975	1	0,255924	1	0,405176	1	0,493891

Pfaffl Method

	hmbs		tbp		ywahz		Mean Ref		Sox2		Drd2		Pcsk2		Thbs2		POMC	
	Hypofyse	Adenoom	Hypofyse	adenoom	hypofyse	Adenoom	hypofyse	adenoom	Hypofyse	Adenoom	Hypofyse	Adenoom	Hypofyse	Adenoom	Hypofyse	Adenoom	Hypofyse	Adenoom
Mean	21,64293	21,58685	24,82434	24,8354	19,60232	19,55844	22,025	21,40766	20,75035	21,58577	21,65799	24,58353	20,69447	22,0128	27,0264	27,77346	17,85131	18,20295
SD	0,328354	0,896464	0,475512	1,021226	0,602922	0,857185	0,395541	1,098036	0,450156	1,263307	0,908402	2,617894	1,023868	2,4394	1,01782	2,378689	1,410515	2,248512
SD/Mean	0,015171	0,041528	0,019155	0,04112	0,030758	0,043827	0,017959	0,051292	0,021694	0,058525	0,041943	0,10649	0,049475	0,110817	0,03766	0,085646	0,079015	0,123525
(SD/mean)^2	0,00023	0,001725	0,000367	0,001691	0,000946	0,001921	0,000323	0,002631	0,000471	0,003425	0,001759	0,01134	0,002448	0,01228	0,00142	0,007335	0,006243	0,015258
?(ref+gene)									0,028163	0,07782	0,045626	0,118199	0,052634	0,122112	0,04172	0,09983	0,08103	0,13375
Mean N-fold									1	-1,76628	1	-8,95586	1	-2,58559	1	-1,67597	1	-1,32014
SD N-fold = ?(ref+gene) * mean N-fold									0,000471	-0,00605	0,001759	-0,10156	0,002448	-0,03175	0,00142	-0,01229	0,006243	-0,02014

Table D3 (continuation)